REMARKS

Claims 61-63, 65-68, 70-81, and 83-103 were pending. Claims 70-76, 78, 82-85, 88-90 and 92 have been canceled Claim 100 has been withdrawn. Claims 61, 77, 87, 95 and 101 have been amended. Claims 104-115 have been added.

Support for the amendments to the claims can be found in previously pending claims 84 and 86 and pending claim 90. Further support for the amendments to the claims can be found, for example, at page 10 to page 11, at page 22 to page 23, at page 45-46, and at page 69 to page 70. Accordingly, no new matter has been added to the application by way of these amendments.

The foregoing claim amendments have been made solely for the purpose of expediting prosecution of the present application and should in no way be construed as an acquiescence to any of the Examiner's rejections in this or in any former Office Action issued in the present application. Applicants reserve the right to pursue the subject matter of the present claims prior to being amended herein in this application or in another related application.

In view of the foregoing claim amendments and the arguments set forth below, Applicants respectfully submit that the claims are now in condition for allowance.

Interview

Applicants thank Examiners Archie and Navarro, and Supervisory Examiner Foley for the courtesy of the interview which took place on November 28, 2007. Applicants appreciate the guidance with respect to possible claim amendments provided by Supervisory Examiner Foley The claim amendments presented herein reflect the discussion that took place during the interview.

The Pending Claims

In some embodiments, pending claims are directed to a composition comprising an amount of a monoclonal antibody effective to treat neonates having a staphylococcal infection and a pharmaceutically acceptable carrier, wherein the antibody specifically binds to poly-glycerol phosphate of Lipoteichoic acid (LTA) of Gram positive bacteria with a binding affinity of about 10^{-6} M or higher and is of the IgG isotype, wherein the antibody binds to and enhances opsonization of multiple serotypes of *Staphylococcus epidermidis,* coagulase negative

staphylocci and *Staphylococcus aureus* by phagocytic cells with or without complement as compared to an appropriate control in an in vitro opsonization assay.

In other embodiments, pending claims are further directed to a composition comprising a monoclonal antibody which specifically binds to poly-glycerol phosphate of LTA of Gram positive bacteria with a binding affinity of 10^{-8} M or higher, or antigen binding fragment thereof, and a pharmaceutically acceptable carrier, wherein the monoclonal antibody comprises the complementarity determining regions (CDRs) of the heavy and light chain variable regions of monoclonal antibody 96-110 set forth as SEQ ID NO:87 and SEQ ID NO:89.

Additionally, the pending claims are directed to a composition comprising a monoclonal antibody which specifically binds to poly-glycerol phosphate of LTA of Gram positive bacteria with a binding affinity of 10^{-8} M or higher, or antigen binding fragment thereof, and a pharmaceutically acceptable carrier, wherein the monoclonal antibody comprises the heavy chain variable region set forth as SEQ ID NO:87.

The pending claims are also directed to a composition comprising a monoclonal antibody which specifically binds to poly-glycerol phosphate of LTA of Gram positive bacteria with a binding affinity of 10^{-8} M or higher, or antigen binding fragment thereof, and a pharmaceutically acceptable carrier, wherein the monoclonal antibody comprises the light chain variable region set forth as SEQ ID NO:89.

The pending claims are also directed to a composition comprising a monoclonal antibody which specifically binds to poly-glycerol phosphate of LTA of Gram positive bacteria with a binding affinity of 10^{-8} M or higher, or antigen binding fragment thereof, and a pharmaceutically acceptable carrier, wherein the monoclonal antibody comprises a heavy chain comprising the heavy chain complementarity determining regions (CDRs) of the monoclonal antibody 96-110 and a variable region having 80% amino acid identity with SEQ ID NO:87.

The pending claims are also directed to a composition comprising a monoclonal antibody which specifically binds to poly-glycerol phosphate of LTA of Gram positive bacteria with a binding affinity of 10^{-8} M or higher, or antigen binding fragment thereof, and a pharmaceutically acceptable carrier, wherein the monoclonal antibody comprises a light chain comprising the light chain complementarity determining regions (CDRs) of the monoclonal antibody 96-110 and a variable region having 80% amino acid identity with SEQ ID NO:89.

The pending claims are also directed to a composition comprising a monoclonal antibody and a pharmaceutically acceptable carrier, wherein the monoclonal antibody i) specifically binds to poly-glycerol phosphate of LTA of Gram positive bacteria with a binding affinity of 10^{-8} M or higher, ii) binds to and enhances opsonization of multiple serotypes of *Staphylococcus epidermidis*, coagulase negative staphylococci, and *Staphylococcus aureus* by phagocytic cells with or without complement as compared to an appropriate control in an in vitro opsonization assay, and iii) comprises a heavy chain comprising the complementarity determining regions (CDRs) of the monoclonal antibody 96-110 heavy chain variable region set forth as SEQ ID NO:87 and having at least 70% amino acid identity with the monoclonal antibody 96-110 heavy chain variable region set forth as SEQ ID NO:87.

The pending claims are also directed to a composition comprising a monoclonal antibody and a pharmaceutically acceptable carrier, wherein the monoclonal antibody i) specifically binds to poly-glycerol phosphate of LTA of Gram positive bacteria with a binding affinity of 10^{-8} M or higher, ii) binds to and enhances opsonization of multiple serotypes of *Staphylococcus epidermidis*, coagulase negative staphylococci, and *Staphylococcus aureus* by phagocytic cells with or without complement as compared to an appropriate control in an in vitro opsonization assay, and iii) comprises a light chain comprising the complementarity determining regions (CDRs) of the monoclonal antibody 96-110 light chain variable region set forth as SEQ ID NO:89 and having at least 70% amino acid identity with the monoclonal antibody 96-110 light chain variable region set forth as SEQ ID NO.89.

Rejection of Rejection of Claims 61, 62, 64-68, 81, 82, 84-86, 89, 90, 92, 93 and 100 Under Section 102(b)

The Examiner has rejected claims 61, 62, 64-68, 81, 82, 84-86, 89, 90, 92, 93 and 100 under §102(b) as being anticipated by Aasjord *et al.* in light of Roitt *et al.* This rejection is respectfully traversed.

As set forth above, in certain embodiments, the pending claims require an amount of a monoclonal antibody effective to treat neonates having a staphylococcal infection and a pharmaceutically acceptable carrier, wherein the antibody specifically binds to poly-glycerol phosphate of Lipoteichoic acid (LTA) of Gram positive bacteria with a binding affinity of about

10^{-8} M or higher and is of the ***IgG isotype***, wherein the antibody binds to and enhances opsonization of multiple serotypes of *Staphylococcus epidermidis*, coagulase negative staphylocci and *Staphylococcus aureus* by phagocytic cells with or without complement as compared to an appropriate control in an in vitro opsonization assay. Other claimed embodiments require that the antibody share structural features (i.e., sequence homology) with the 96-110 monoclonal antibody.

For the reasons stated below, it is Applicants' position that the Examiner has failed to establish a *prima facie* case of anticipation. "Anticipation requires a showing that each limitation of a claim is found in a single reference, either expressly or inherently." *Perricone v. Medicis Pharm. Corp.*, 432 F.3d 1368, 1376 (Fed. Cir 2005).

Aasjord discloses two ***IgM*** monoclonal antibodies that bind to LTA. In addition, the antibodies are not shown to have any of the functional or structural limitations required by the pending claims. Specifically, Aasjord fails to teach or suggest a monoclonal antibody which binds to and enhances opsonization of multiple serotypes of *Staphylococcus epidermidis*, coagulase negative staphylococci and *Staphylococcus aureus* by phagocytic cells with or without complement as compared to an appropriate control in an in vitro opsonization assay, and wherein said monoclonal antibody is present in amount effective to treat neonates having a staphylococcal infection. Aasjord also fails to teach or suggest a monoclonal antibody, or antigen binding fragment thereof, wherein the monoclonal antibody shares structural features of the 96-110 MAB.

Accordingly, because Aasjord *et al.*, fails to teach or suggest each and every element of the presently claimed invention, applicants respectfully request that the rejection under 35 U S C. §102(b) be reconsidered and withdrawn.

Rejection of Claims 69-76, 91 and 95
Under Section 102(b)

The Examiner has rejected claims 69-76, 91 and 95 under §102(b) as being anticipated by Takada *et al.* This rejection is respectfully traversed.

As set forth above, in certain embodiments, the pending claims require an amount of a monoclonal antibody effective to treat neonates having a staphylococcal infection and a pharmaceutically acceptable carrier, wherein the antibody specifically binds to poly-glycerol

phosphate of Lipoteichoic acid (LTA) of Gram positive bacteria with a binding affinity of about 10^{-8} M or higher and is of the ***IgG isotype***, wherein the antibody binds to and enhances opsonization of multiple serotypes of *Staphylococcus epidermidis,* coagulase negative staphylocci and *Staphylococcus aureus* by phagocytic cells with or without complement as compared to an appropriate control in an in vitro opsonization assay. Other claimed embodiments require that the antibody share structural features (i.e., sequence homology) with the 96-110 monoclonal antibody.

Takada discloses an ***IgM*** monoclonal antibody that binds to LTA (see page 64, left column, second paragraph). In addition, the antibody is not shown to have any of the functional or structural limitations required by the pending claims. Specifically, Takada fails to teach or suggest a monoclonal antibody which binds to and enhances opsonization of multiple serotypes of *Staphylococcus epidermidis,* coagulase negative staphylococci and *Staphylococcus aureus* by phagocytic cells with or without complement as compared to an appropriate control in an in vitro opsonization assay, and wherein said monoclonal antibody is present in amount effective to treat neonates having a staphylococcal infection. Takada also fails to teach or suggest a monoclonal antibody, or antigen binding fragment thereof, wherein the monoclonal antibody shares structural features of the 96-110 MAB.

Accordingly, because Takada *et al.*, fails to teach or suggest each and every element of the presently claimed invention, applicants respectfully request that the rejection under 35 U.S.C. §102(b) be reconsidered and withdrawn.

Rejection of Claim 94
Under Section 102(b)

The Examiner rejects claim 94 under §102(b) as being anticipated by Chugh *et al.* The Examiner also rejects claim 94 under 35 U.S.C. §102(b) as being anticipated by West *et al.*

As set forth above, in certain embodiments, the pending claims are directed to ***monoclonal antibodies*** effective to treat neonates having a staphylococcal infection and a pharmaceutically acceptable carrier, wherein the antibody specifically binds to poly-glycerol phosphate of Lipoteichoic acid (LTA) of Gram positive bacteria with a binding affinity of about 10^{-8} M or higher and is of the IgG isotype, wherein the antibody binds to and enhances opsonization of multiple serotypes of *Staphylococcus epidermidis,* coagulase negative

staphylocci and *Staphylococcus aureus* by phagocytic cells with or without complement as compared to an appropriate control in an in vitro opsonization assay. Other claimed embodiments require that the antibody share structural features (i.e., sequence homology) with the 96-110 monoclonal antibody.

The Chugh and West references, which each disclose polyclonal antibodies, fail to teach or suggest each and every element of the presently claimed invention. Specifically, each of the references disclose polyclonal antibodies and fail to teach any ***monoclonal*** anti-LTA antibody, let alone any monoclonal anti-LTA antibody having the functional or structural properties presently claimed Applicants respectfully request that the rejection under 35 USC § 102(b) be reconsidered and withdrawn.

Rejection of Claims 61, 62, 64-67, 81-90, 92, 93 and 100 Under Section 102(b)

The Examiner has rejected claims 61, 62, 64-67, 81-90, 92, 93 and 100 under §102(b) as being anticipated by Hamada *et al.* in light of Roitt *et al.* This rejection is respectfully traversed.

As set forth above, the pending claims are directed to compositions comprising ***an amount of a monoclonal antibody effective to treat neonates having a staphylococcal infection*** and a pharmaceutically acceptable carrier, wherein the antibody specifically binds to poly-glycerol phosphate of Lipoteichoic acid (LTA) of Gram positive bacteria ***with a binding affinity of about 10^{-8} M or higher*** and is of the IgG isotype, wherein the antibody ***binds to and enhances opsonization of multiple serotypes of Staphylococcus epidermidis, coagulase negative staphylocci and Staphylococcus aureus by phagocytic cells with or without complement*** as compared to an appropriate control in an in vitro opsonization assay.

The Hamada reference describes anti-LTA monoclonal antibody 3G6. As set forth in more detail below, not all monoclonal antibodies, let alone monoclonal antibodies with binding specificity to LTA, enhance opsonization of bacteria, let alone enhance opsonization of multiple serotypes of both coagulase positive and coagulase negative, *i.e.*, *Staphylococcus epidermidis* and *Staphylococcus aureus*, with or without complement.

The Examiner states that the 3G6 antibody disclosed by Hamada *et al.* would inherently opsonize gram positive bacteria in light of the teaching of Roitt However, the Examiner's statement that "antibodies inherently have the ability to opsonize bacteria by virtue of their binding" is incorrect with respect to anti-LTA antibodies Under principles of inherency, "if the

As inherency may not be established by probabilities or possibilities, Applicants respectfully submit that the claimed functional properties are not inherent in all antibodies, and specifically not inherent in the antibodies in the cited art

Moreover, the Hamada *et al.* reference itself casts doubt on the ability of the 3G6 antibody disclosed therein to bind to poly-glycerol phosphate of Lipoteichoic acid (LTA) of Gram positive bacteria with a binding affinity of about 10^{-8} M or higher or to bind to and enhance opsonization of multiple serotypes of *Staphylococcus epidermidis,* coagulase negative staphylococci and *Staphylococcus aureus* by phagocytic cells with or without complement as compared to an appropriate control in an in vitro opsonization assay, or to be present in an effective amount to treat neonates having a staphylococcal infection. The reference provides aggregation data showing the reactivity of the 3G6 monoclonal antibody with various strains of bacteria As the aggregation data show, the 3G6 monoclonal antibody aggregates or agglutinates some but not all species and serotypes of Gram positive bacteria and more specifically, some but not all strains of staphylococci. Hamada *et al.* notes that certain Gram positive bacteria, more specifically some strains of staphylococci, were not agglutinated by 3G6 and postulate that this lack of agglutination indicates that the antibody binds to an epitope exposed to a different degree on the outermost layer of different bacterial cells (see p. 1020 of the reference). Thus, the 3G6 antibody appears to bind to an epitope which is not accessible on all bacterial species. It is known in the art, however, that antigen accessibility is critical for an antibody to effectively protect, *i.e.*, opsonize, against bacterial infection (see, *e.g.*, Gor *et al.* Infect Immun 2005 Mar;73(3):1304-12, attached as Appendix D). Gor *et al.* report that mice with antibodies to PsaA or PpmA did not provide protection, whereas mice with antibodies to PspA or type 3 PS provided protection from systemic challenge with type 3 pneumococci. Gor *et al.* confirmed that while PspA was readily detectable on the surface of pneumococci, *PsaA and PpmA were not readily detectable on the surface*, thus indicating that antigen accessibility is critical for an antibody to effectively protect, *i.e.*, opsonize. Gor *et al.* concludes that suitable candidates for vaccines are antibody-accessible antigens capable of supporting opsonization. Thus, the 3G6 antibody appears to bind to an epitope which may not be sufficiently accessible to support opsonization of the majority of strains of staphylococci.

Further, the heterogeneity in functional activity displayed by the 3G6 antibody indicates that the antibody is not appropriate for therapeutic use, *e.g.*, for treating neonates having

prior art necessarily functions in accordance with, or includes, the claimed limitations, it anticipates." *Mehl/Biophile Int'l Corp. v. Milagraum,* 192 F.3d 1362, 1365 (Fed Cir 1999). To show that the prior art "necessarily" functions in accordance with, or includes the claimed limitations, one must show more than a mere probability or possibility of the inherent feature's existence. *See SmithKline Beecham Corp. v. Apotex Inc.*, 403 F.3d 1331, 1346 (Fed. Cir. 2005). Therefore, "[i]nherency... ***may not be established by probabilities or possibilities***. The mere fact that a certain thing ***may*** result from a given set of circumstances is not sufficient." *Mehl/Biophile,* 192 F.3d 1362 at 1365 (emphasis added) (quoting *Hansgirg v. Kemmer,* 102 F.2d 212, 214 (CCPA 1939)).

As set forth in more detail below, it cannot be established that *all* monoclonal antibodies are opsonic and consequently protective.[1] As set forth in the Amendment and Response filed on August 23, 2007, at the time the application was filed, the art taught that anti-LTA antibodies were not opsonic. Takeda *et al.* published that antibodies to teichoic acid afforded no protection against bacteremia, whereas antibodies to PS/A effectively protected against bacteremia (Circulation 86(6):2539-2546 (1991); attached as Appendix C). See, *e.g.*, page 2542-43 of the reference which shows that immunization with *S. epidermidis* strain SE360 which expresses a teichoic acid (see page 2540), used as a control, provided no protection against bacterial endocarditis. Kojima *et al.* (Journal of Infectious Diseases 162.435-441 (1990); attached as Appendix B) similarly report that antibodies to teichoic acid, actually used as a control, afforded no protection against bacteremia See, *e.g.*, page 438 of the reference which shows that immunization with *S. epidermidis* strain SE360 which expresses a teichoic acid (see page 436), used as a control, in fact, provided no protective efficacy on dissemination of coagulase-negative staphylococci from an infected catheter. Further, Fattom *et al.* show that anti-teichoic acid antibodies, used as a control, lacked opsonophagocytic activity, whereas antibodies to capsular type 1 and type 2 exhibited opsonophagocytic activity (J. Clin. Micro. 30(12):3270-3273 (1992), attached as Appendix A). See, *e.g.*, Figure 2 on page 3272 of the reference which shows that anti-teichoic acid antibodies were used as controls and showed no opsonophagocytic activity Thus, the functional properties of the presently pending claims are not inherent in all antibodies.

[1] Opsonic activity of an antibody is predictive of the ability of an antibody to confer protection (see *e.g.*, Henckaerts *et al.*, Vaccine 2007 Mar 22;25(13):2518-27)

staphylococcal infections As many different strains of Staphylococci are typically isolated from individual neonates, in order to be suitable for treatment of neonates having a staphylococcal infection as required by pending claim 61, an antibody would have to have uniform binding and opsonization characteristics for the majority of strains of Staphylococci. As evidenced by the aggregation data, the 3G6 antibody does not have such uniform properties.

In addition, the Hamada *et al.* fails to teach or suggest a monoclonal antibody which binds to poly-glycerol phosphate of LTA ***with a binding affinity of about 10^{8} M or higher.*** The reference also fails to teach or suggest a composition comprising an amount of a monoclonal antibody ***effective to treat neonates having a staphylococcal infection.*** The Hamada *et al.* reference also fails to teach or suggest a monoclonal antibody having the structural properties required by claim 77, the claims that depend therefrom, and new claims 104-115.

Roitt does not make up for the deficiencies of the primary reference. The Examiner relies on Roitt as teaching that "antibodies inherently have the ability to opsonize bacteria by virtue of their binding...to a large extent as compare [sic] to the absence of any opsonin (see page 16 of the Office Action dated February 23, 2007). However, as set forth above and as illustrated by the references supplied with this Amendment and Response, not all antibodies have the ability to opsonize bacteria.

Accordingly, because Hamada *et al.*, fails to teach or suggest each and every element of the presently claimed invention with certainty, applicants respectfully request that the rejection under 35 U S C. §102(b) be reconsidered and withdrawn.

Rejection of Claim 94
Under Section 103(a)

The Examiner has rejected claim 94 under §103(a) as being unpatentable over Aasjord *et al.* in view of Hamada *et al.* and in view of Schwarzberg. This rejection is respectfully traversed.

The test for *prima facie* obviousness is consistent with the legal principles enunciated in *KSR Int'l Co. v. Teleflex Inc.*, 127 S Ct 1727 (2007). *Takeda Chem. Indus., Ltd. v. Alpharma Pty., Ltd.*, 2007 U.S. App. LEXIS 15349, at *13 (Fed Cir. 2007). "While the *KSR* Court rejected a rigid application of the teaching, suggestion, or motivation ("TSM") test, the Court acknowledged the importance of identifying 'a *reason* that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention

does' in an obviousness determination." *Id.* at *13-14 (quoting *KSR*, 127 S. Ct. at 1731) (emphasis added). Although the TSM test should not be applied in a rigid manner, it can provide helpful insight to an obviousness inquiry *KSR*, 127 S. Ct. at 1731. The *KSR* Court upheld the secondary considerations of non-obviounsess, noting that there is "no necessary inconsistency between the idea underlying the TSM test and the *Graham* analysis." *Id.*

The subject matter of the pending claims is set forth above. As set forth above, Aasjord *et al.* fail to teach or suggest anti-LTA antibodies of an IgG isotype. Hamada et al. fail to teach or suggest anti-LTA antibodies having the claimed functional characteristics and Schwarzberg fails to make up for this deficiency. The Examiner relies on Schwarzberg for the teaching that fragments retain a high degree of specificity and affinity. However, the only claims that read on fragments of antibodies require a degree of structural similarity to the 96-110 antibody. Antibodies having structural similarity to the 96-110 antibody are not taught or suggested by any of the art of record.

In summary, Applicants respectfully request that the rejection of these claims in view of Aasjord *et al.*, Hamada *et al.* and Schwarzberg be reconsidered and withdrawn on the grounds that there is *no reason to combine* the teaching of Aasjord *et al.*, Hamada *et al.* and Schwarzberg Notwithstanding the lack of reason to combine the cited references, the references, alone or in combination, do not teach or suggest each and every element of the claimed invention

Rejection of Claim 95
Under Section 103(a)

The Examiner has rejected claim 95 under §103(a) as being unpatentable over Takada *et al.* in view of Hamada *et al* This rejection is respectfully traversed.

Claim 95 is directed to a pharmaceutical composition comprising an effective amount of an antibody of claim 77, for use in a human neonate Claim 77 requires that the antibody has the CDRs of the heavy and light chain variable regions of the monoclonal antibody 96-110 set forth as SEQ ID NO:87 and SEQ ID NO:89.

As set forth above, Takada *et al.* and Hamada *et al.* fail to teach or suggest an antibody having the required degree of structural similarity to the 96-110 antibody.

In summary, Applicants respectfully request that the rejection of these claims in view of Takada *et al.* and of Hamada *et al.* be reconsidered and withdrawn on the grounds that there is *no*

reason to combine the teaching of Takada *et al.* and of Hamada *et al.* Notwithstanding the lack of reason to combine the cited references, the references, alone or in combination, do not teach or suggest each and every element of the claimed invention.

SUMMARY

If a telephone conversation with Applicants' Attorney would expedite the prosecution of the above-identified application, the Examiner is urged to call Applicants' Attorney at (617) 227-7400.

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Respectfully submitted,

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Appendix A

JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1992, p. 3270–3273
0095-1137/92/123270-04$02.00/0

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Capsular Polysaccharide Serotyping Scheme for *Staphylococcus epidermidis*

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A scheme for the capsular typing of *Staphylococcus epidermidis* that is based on direct slide agglutination between proteinase-treated bacterial cells and specific antisera is described. Antisera were prepared from serum from rabbits immunized with two selected strains of encapsulated *S. epidermidis* isolated from bacteremic patients. Antisera were shown to be type specific and designated type 1 and type 2. Blood isolates of *S. epidermidis* from hospitals in different locations within the United States and Europe were serotyped, and it was found that over 90% of all strains were of type 1 or type 2. Type-specific antibodies mediated type-specific opsonophagocytosis and killing of *S. epidermidis*. The specificity was shown to be due to two distinct capsular polysaccharides. The data presented in this report may open a new window on the pathogenesis of *S. epidermidis* which could lead to the development of new vaccines and therapies.

For a long time, coagulase-negative staphylococci, especially *Staphylococcus epidermidis*, were recognized as normal skin commensals and were considered contaminants rather than true pathogens. Bacteremia due to this organism was attributed to skin carriage (17). In recent years, *S. epidermidis* has emerged as a leading cause of nosocomial infections (18). Immunocompromised neonates, patients undergoing chemotherapy, and other patients with indwelling medical devices are at high risk for contracting *S. epidermidis* bacteremia (5–7, 11, 12, 14). A recent report indicated that such epidemics can often be traced to medical personnel (1).

Slime production and secretion were suggested as major mechanisms facilitating adherence to catheters and other medical devices, thus shielding the bacteria from being phagocytosed by polymorphonuclear leukocytes (PMNs) (2–4). These reports suggested that slime was a virulence factor and a possible protective antigen. However, data on slime as a virulence factor in vivo are still equivocal. Recently, Kotilainen showed that adherence and slime production by *S. epidermidis* did not correlate with virulence; half of the septicemic cases were caused by non-slime producers (10). Moreover, a recent report by Patrick et al. (13) showed that slime production did not increase the infectivity and bacteremic occurrence of *S. epidermidis* compared with those of non-slime-producing isolates, despite the fact that slime-producing isolates were more adherent to catheters. Different surface components, such as capsular polysaccharide-adhesin (9, 20), or 200- to 220-kDa protein, were proposed as adherence factors for *S. epidermidis* (19). The significant findings that active immunization with the capsular polysaccharide-adhesin protected animals against challenge with the homologous strain and that antibodies to the capsular material mediated type-specific phagocytosis suggest that the pathogenesis of *S. epidermidis* may be similar to that of other encapsulated gram-positive cocci, i.e., *Staphylococcus aureus* and *Streptococcus pneumoniae* (8, 16).

In this report, we present a scheme for serotyping blood isolates of *S. epidermidis* and other significant clinical isolates. This scheme is based on immunological identification of capsular polysaccharide surface antigens.

Vaccines for the production of typing sera were prepared from two blood isolate prototype strains, designated strain 526 (type 1) and strain 548 (type 2). Their identification as *S. epidermidis* was confirmed in our laboratory by using API STAPH Trac (API Analytab Products, Division of Sherwood Medical, Plainview, N.Y.) and the coagulase test (Remel Laboratories, Lenexa, Kans.). Both prototypes were shown to be encapsulated as evidenced by two criteria, resistance to in vitro phagocytosis by PMNs in the presence of anti-teichoic acid serum and lack of agglutination with anti-glyceral-teichoic acid serum. Strains were grown on Columbia medium (Difco Laboratories, Detroit, Mich.) agar plates supplemented with 4% NaCl (CSA) under 5% CO_2 at 37°C for 18 h. Cells were washed off the agar plates with 20 ml of 3% formalinized phosphate-buffered saline (PBS), pH 7.2. After dispersion of cell clumps by gentle mixing with a glass rod, the suspension was centrifuged and the pellet was resuspended in PBS, washed once, and resuspended in 0.5% formalinized PBS at a final concentration that gave an optical density reading of 0.6 at 500 nm in a 1.0-ml cuvette. Viability checks were made with CSA plates that were incubated at 37°C. In addition, cell suspensions were subjected to direct cell agglutination tests against anti-teichoic acid serum. A titer of 20 was considered to be indicative of encapsulation, and vaccines with this titer were stored at 4°C. New Zealand White rabbits weighing 6 lb (ca. 3 kg) were immunized with prototype vaccines. A quantity of 10 ml of preimmune blood was obtained and tested against purified teichoic acid. Rabbits considered normal were subsequently immunized as follows. During the first week, a 0.1-ml subcutaneous injection was followed by two 0.1-ml intravenous injections. Thereafter, the animals were immunized intravenously three times a week with doses of 0.2 ml, followed by 0.3 ml the next week and finally 0.4 ml for the subsequent week. Five days after the last injection, blood samples were taken and tested by the direct cell agglutination test employing homologous vaccines. When agglutination titers were 1,280 or higher, the animals were exsanguinated and sera were collected and stored at 4°C under sterile conditions.

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Nonencapsulated strain 90 and antiserum-absorbing strains were grown in 1 liter of trypticase soy broth and incubated at 37°C for 18 h with constant aeration. Cells were killed by being heated at 70°C for 4 h and then centrifuged and resuspended in 100 ml of PBS. The resulting suspension was treated with 10 to 20 μg of trypsin (Sigma) per ml and incubated at 37°C (water bath) for 2 h in the presence of a small amount of chloroform. Subsequently, the treated cells were centrifuged, washed three times with PBS, and finally packed in graduated Corex centrifuge tubes. Antisera were absorbed by using 1 volume of packed cells per 2 volumes of whole serum and gently stirring with a glass rod. After complete dispersal of cell clumps, the suspension was stored at 4°C overnight and subsequently centrifuged and the serum was decanted and stored in 0.02% sodium azide at 4°C. Residual teichoic acid antibodies in absorbed typing sera were measured by the direct cell agglutination method, with nonencapsulated strain 90.

Whole-cell antigens for the agglutination procedures were prepared as follows. Bacteria were inoculated on CSA plates at 37°C for 18 h under 5% CO_2 tension. Cells were collected by centrifugation, washed in PBS, and resuspended in 5 ml of PBS. The cell suspension was then treated with proteinase (Sigma) at a concentration of 10 to 20 μg/ml. After 2 h at 37°C, cells were collected by centrifugation, washed in PBS, resuspended in 10 ml of PBS with 3% formalin, dispersed with a Vortex mixer, and incubated at room temperature for 18 h. The cells were washed once with PBS and resuspended in 10 ml of PBS, and the density of the suspension was adjusted to an optical density at 550 nm of 0.6 to 1.

Twofold dilutions of antiserum in PBS were prepared. A 5-μl sample of each serum dilution was placed onto a microscope slide and mixed with a wooden toothpick with 5 μl of cell suspension. Agglutination was determined visually after 1 min.

The slide agglutination test showed that strain 526, the type 1 strain, and strain 548, a type 2 strain, were type specific as evidenced by the agglutinability of cells only with the homologous bacteria. The antiserum-agar technique (15) revealed that the type specificity of the antisera was based on surface capsular antigens. As shown in Fig. 1, strain 526 (type 1) grown on homologous serum-agar plates (Columbia agar plus 5% specific serum) secreted capsular antigens which formed a precipitin halo around colonies of type 1 cells. Strain 526 grown on type 2 serum-agar plates was negative for a precipitin halo. Strain 480 (type 2) did not show a distinct halo in either homologous or heterologous serum-agar plates. Accordingly, the capsular polysaccharides were prepared primarily from growth medium of type 1 and cell paste of type 2.

The surface nature of the type-specific antigens of types 1 and 2 was confirmed. Cell preparations of type 1 and type 2 cells were autoclaved for 20 min at 121°C to remove capsular antigens and subsequently subjected to agglutination with anti-teichoic acid serum. Autoclaved cells lost their agglutinating activity, indicating that autoclaving removes appreciable levels of type-specific antigens and results in extensive cross-reactivity between treated cells and heterologous antiserum and anti-teichoic acid serum (data not shown). These observations suggest that the type specificity of this typing scheme for *S. epidermidis* is dependent on a surface antigen or capsules. Preliminary results, including reduction of carboxyl groups, migration in an electrical field, and liquid chromatography of hydrolyzed polysaccharides, suggest that these capsules are acidic polysaccharides consisting of aminouronic acids and amino sugars. In vitro phagocytosis

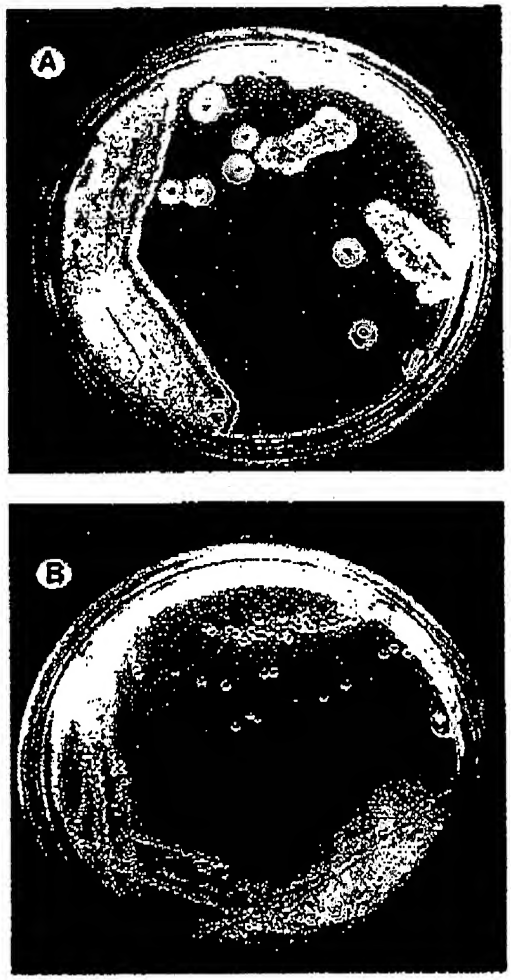

FIG. 1. *S. epidermidis* type 1 and type 2 growth on Columbia agar plates containing rabbit type 1 or type 2 antiserum. Strains 526 and 480 were streaked on Columbia agar plates containing the appropriate rabbit antiserum and incubated overnight at 37°C in a 5% CO_2 atmosphere. Strain 526 (type 1) was inoculated onto plate A containing 5% rabbit anti-type 1 serum, and strain 480 (type 2) was streaked onto plate B containing 5% rabbit anti-type 2 serum.

studies support the notion that these capsules are type specific and can impede phagocytosis by PMNs. Figure 2 shows that homologous type 1 serum was effective in opsonizing type 1 cells but not type 2 cells. Type 2 antiserum was effective in opsonizing homologous cells only.

Purified capsular polysaccharides from type 1 and type 2 cells were run in immunodiffusion (Fig. 3). Type 1 gave a line of precipitation with anti-type 1 sera only. Type 2 polysaccharide gave one line of precipitation with type 2 antisera. These results indicate that the typing sera are specific to the capsules and that these are two distinct non-cross-reactive capsular polysaccharides. Moreover, other copurifying polysaccharides reacted with both type 1 and type 2 antisera,

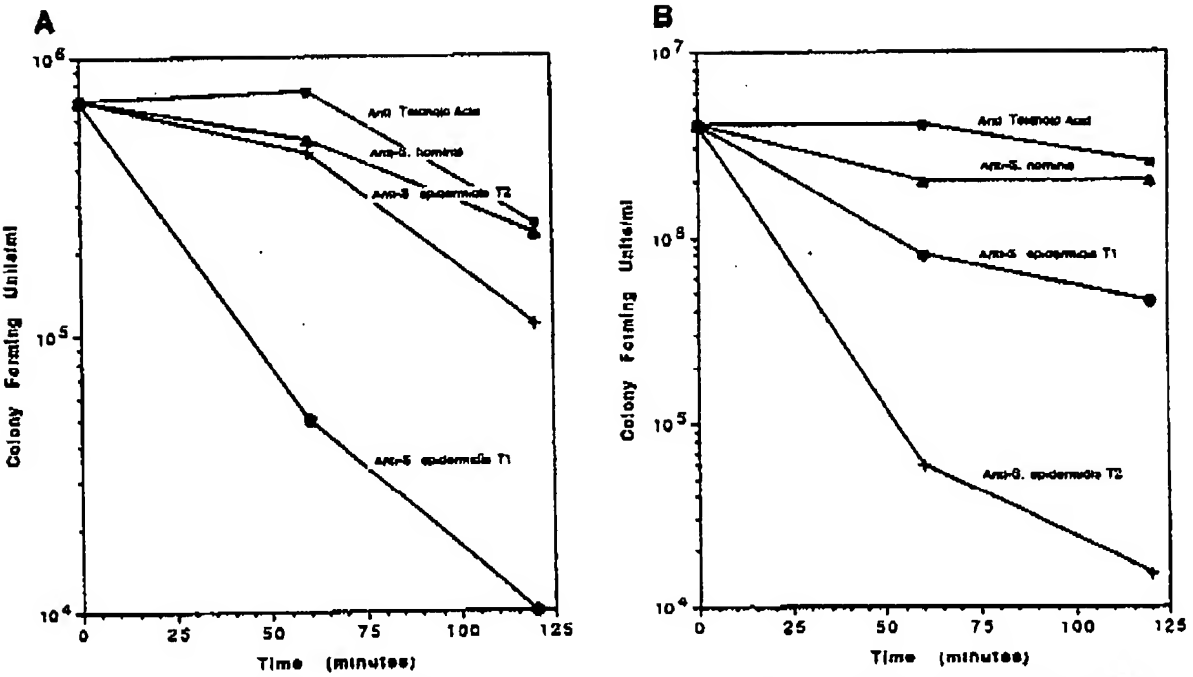

FIG. 2. Opsonophagocytic activity of *S. epidermidis* type 1 (A) and type 2 (B) rabbit antibodies. The reaction mixture contained 10^6 human PMNs, ~10^6 organisms (strain 526 for type 1 and strain 480 for type 2), and 10% serum. The reaction was carried out at 37°C with gentle rocking. Aliquots were removed at 60 and 120 min, diluted in H_2O, and plated on Columbia agar plates. Viable counts were recorded after 24 h of incubation at 37°C and 5% CO_2. Anti-teichoic acid and anti-*Staphylococcus hominis* were used as controls.

indicating the possible presence of shared polysaccharide antigens for all *S. epidermidis* organisms which may be referred to as common antigens. These data may explain the contradicting results regarding *S. epidermidis* serotyping (9).

Using these sera and serum from a nonencapsulated strain, we typed *S. epidermidis* clinical isolates from different hospitals within the United States and Europe. Data shown in Table 1 demonstrate that more than 90% of bacteremic isolates were of either type 1 or type 2. Of the isolates, 77% were of type 2 and 14% were of type 1. The remaining nontypeable 9% could be of other capsular types. This distribution was found also with other clinical isolates from catheter- and other medical device-related infections (data not shown). Further studies have been initiated for the chemical characterization of these capsules. Moreover, since antibodies to these capsules mediated opsonophagocytosis and killing of the bacteria by human PMNs, we are investigating the possibility of using these polysaccharides as vaccines for passive or active immunization.

Bacterial isolates were kindly supplied by Frida Stock and Vee J. Gill from the Microbiology Service, NIH, Bethesda, Md.; William Bartholomew, VA Medical Center, Kansas City, Mo.; Jennifer Susan Daly, The Medical Center of Central Massachusetts, Worcester, Mass.; and Ian Philips, Department of Microbiology, St. Thomas Hospital, London, United Kingdom. Joan Brisker confirmed the identification of the isolates. We are grateful to John B. Robbins.

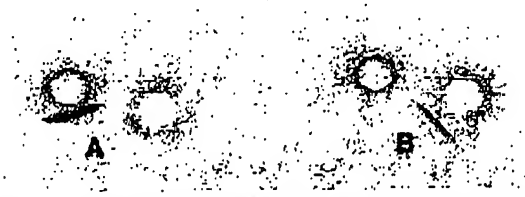

FIG. 3. Double immunodiffusion of *S. epidermidis* capsular polysaccharides. Purified capsular polysaccharides (0.5 mg/ml) were placed in the central wells (type 1, left, and type 2, right). Rabbit anti-type 1 and anti-type 2 antisera were added to wells A and B, respectively. The plates were incubated overnight at 4°C.

TABLE 1. Capsular types of *S. epidermidis* bacteremic isolates from patients

Location	No. of isolates			
	Total	Type 1	Type 2	Nontypeable
Bethesda, Md.[a]	29	3	23	3
Kansas City, Mo.[b]	13	0	12	1
Worcester, Mass.[c]	10	1	8	1
London, United Kingdom[d]	39	9	27	3
Total (%)	91 (100)	13 (14)	70 (77)	8 (9)

[a] Clinical Center, National Institutes of Health
[b] Veterans Administration Medical Center
[c] The Medical Center of Central Massachusetts.
[d] St. Thomas Hospital.

Rachel Schneerson, Scott Winston, and Robert Edelman for their support and invaluable suggestions.

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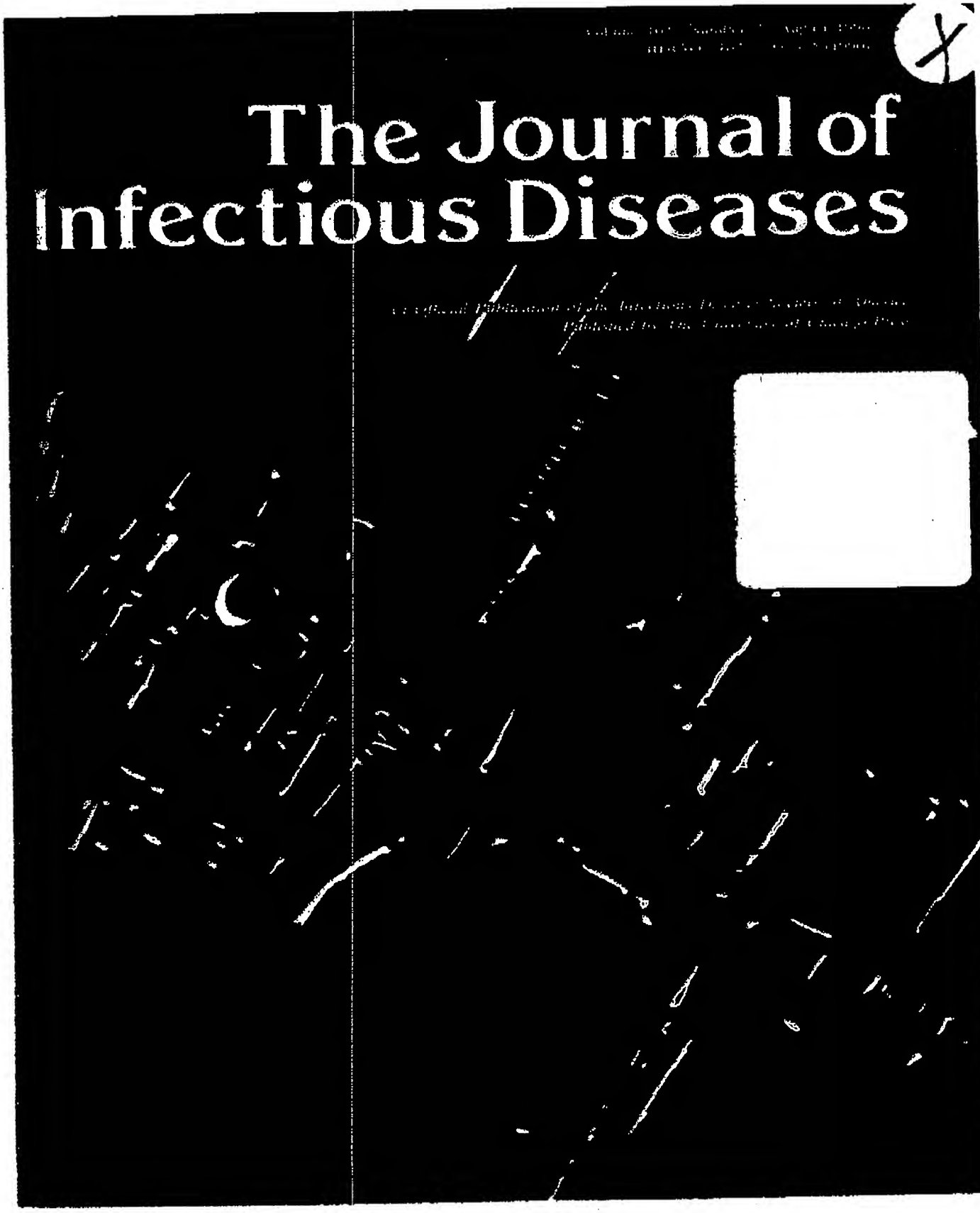
Appendix B



Antibody to the Capsular Polysaccharide/Adhesin Protects Rabbits against Catheter-Related Bacteremia Due to Coagulase-Negative Staphylococci

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A rabbit model of catheter-related bacteremia was developed to study immunity to the capsular polysaccharide/adhesin (PS/A) of coagulase-negative staphylococci. Catheters colonized by coagulase-negative staphylococci were inserted into the right jugular vein and attached to a subcutaneous osmotic pump, and blood cultures were obtained over 14 days. Nonimmune rabbits were bacteremic for 6–8 days after infection, hypoglycemic, and hyperlipidemic and had strong immune responses to teichoic acid but not to PS/A. PS/A immunization, but not teichoic acid immunization, reduced the number of bacteremic days by ~60%, diminished the hypoglycemia and hyperlipidemia, and ablated the immune responses to teichoic acid. Passive infusion of PS/A-specific polyclonal and monoclonal antibodies using a separate, noninfected catheter-pump combination implanted in the left jugular protected against both bacteremia and hematogenous colonization of this contralateral catheter.

In recent years numerous investigators have commented on the apparent increase in the incidence of coagulase-negative staphylococcal infections associated with intravascular catheters and prosthetic devices [1–4]. Coagulase-negative staphylococci are major components of the normal skin flora and are virtually incapable of producing infection in the absence of a foreign body. In the presence of a foreign body, however, they are formidable pathogens.

This unique association between coagulase-negative staphylococci and foreign bodies suggests that coagulase-negative staphylococci have a special ability to adhere to and colonize plastics and other prosthetic materials. In studying this critical first step in the pathogenesis of coagulase-negative staphylococcal infection, attention has focused on the surface properties of these organisms that might mediate attachment to foreign bodies. Some strains of coagulase-negative staphylococci, particularly isolates from foreign body infections, elaborate an extracellular "slime" [5], but the precise role of this complex material in mediating adherence remains unclear despite considerable investigation.

We previously described a capsular polysaccharide purified from a slime-producing strain of *Staphylococcus epidermidis*, RP62A, which appears to be the principal coagulase-negative staphylococcal foreign body adhesin [6]. In vitro, purified PS/A and antibody raised to PS/A inhibited adherence of homologous and heterologous adhesin-positive coagulase-negative staphylococci strains to silicon-elastomer catheter tubing in a dose-response fashion [6]. Most clinical isolates of the staphylococci were found to produce an adhesin serologically indistinguishable from the polysaccharide purified from strain RP62A [7].

These observations suggest that immunotherapy directed specifically against PS/A might have a role in the prevention of coagulase-negative staphylococcal infections by inhibiting attachment of bacteria to foreign bodies. However, the pathogenesis of coagulase-negative staphylococcal infections undoubtedly is complex, involving initial attachment, durable colonization, bacterial multiplication, and, ultimately, invasion of the bloodstream. Indeed, catheter colonization resulting in coagulase-negative staphylococcal bacteremia occurs in only a minority of patients with infected catheters, indicating that colonization is necessary, but not sufficient, for disease [8–10]. Thus it is reasonable that a focus for disease prevention might be interference with bloodborne dissemination after coagulase-negative staphylococci have become established on the foreign body surface, not just inhibition of the attachment process itself.

To investigate these aspects of immunotherapy we developed a rabbit model of intravascular catheter infection, which we used to determine whether antibody against polysaccharide/adhesin (PS/A) can protect animals against coagulase-negative staphylococcal colonization and bacteremia. We also evaluated preliminary evidence regarding enhanced opsonophagocytic killing of coagulase-negative staphylococci.

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Experiments involving human leukocytes were performed under a protocol approved by the Brigham and Women's Hospital Committee for the Protection of Human Subjects from Research Risks.

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Materials and Methods

Bacterial strains and antigens. *S. epidermidis* strains RP62A, RP12, and SE360 have been described by others [5, 11]. Although our previous report [6] indicated that strain RP12 did not produce a PS/A serologically identical to that of RP62A, further data collected during the purification of the RP12 PS/A indicated it was serologically identical to the PS/A of strain RP62A. The inability to detect PS/A production by strain RP12 appeared to be due to interfering substances in the crude extracts originally used to characterize this strain. *S. epidermidis* strain SE360 expresses a teichoic acid (TA) and several uncharacterized surface proteins serologically identical to that of strain RP62A, but fails to elicit antibody to PS/A (see below). *S. epidermidis* strain M33, a clinical isolate from a patient undergoing chronic peritoneal dialysis, was provided by Dr. Edward S. Eisenberg (Montefiore Medical Center, Bronx, NY). This strain was determined to be highly adherent to silastic catheters in vitro [6], but lacked detectable production of PS/A serologically related to that of strain RP62A. Capsular polysaccharide/adhesin from strain RP62A was prepared as described [6].

Polyclonal and monoclonal antibodies and assays for antibodies. Rabbits were hyperimmunized twice weekly for 3 weeks using subcutaneous injections of 100 μg of PS/A from strain RP62A emulsified in complete Freund's adjuvant. Antisera were obtained from the ear artery and tested for antibody to PS/A and purified TA using either an ELISA or immunodiffusion as described [12]. The ELISA uses purified PS/A or TA [6] at a concentration of 10 μg/ml in 0.04 *M* phosphate buffer, pH 7.0, to sensitize plates for 3 h at 37°C, then overnight at 4°C, after which plates are blocked for 1 h at 37°C, then overnight at 4°C with 5% pasteurized skim milk. Addition of antisera and conjugates followed standard protocols using phosphate-buffered saline (PBS) with 0.05% Tween 20 and 5% skim milk.

Conjugates were usually the appropriate antiimmunoglobulin coupled to alkaline phosphatase and reactions were read after 30–90 min at 37°C. After immunization, sera were titered using dilutions of pre- and postimmunization sera starting at a dilution of 1:100. After infection, sera were tested at dilutions of 1:10 or 1:100 only and compared statistically with the serum sample taken just before implantation of the infected catheter. Individual sera were measured in triplicate, and means for groups of similarly treated animals represent the means derived from the means of the individual triplicate samples.

Monoclonal antibodies were prepared using standard techniques [13] after hyperimmunization of BALB/c mice with whole, heat-killed cells of *S. epidermidis* strain RP62A. We used one PS/A-specific clone, designated PS-1, which yielded an IgM antibody, in the passive therapy studies. The antibody was obtained from ascites fluid by ammonium sulfate precipitation and dialysis against PBS.

Infection of rabbits to establish coagulase-negative staphylococcal bacteremia. Silicone elastomer catheters (2.8 French, GESCO International, San Antonio, TX) were dipped for 15 min in a suspension of 10^8 cfu/ml of *S. epidermidis* strain RP62A and then immediately inserted into the right jugular vein of rabbits anesthetized with ketamine (40 mg/kg Ketalar; Parke-Davis, Morris Plains, NJ), and xylazine (10 mg/kg, Rompun; Mobay, Shawnee, KS). For insertion, the neck area was shaved and disinfected with iodine, and a 3-cm incision was made to expose the jugular. A cut-down procedure was then performed and the infected end of the catheter inserted 4–5 cm into the vein. The proximal end of the tubing was attached to a subcutaneous osmotic pump (Alzet model 2ML1; Alza, Palo Alto, CA) filled with 2 ml of 10,000 units/ml heparin, which was delivered continuously for 7 days at a flow of ~10 μl/h. The ear was disinfected with iodine and 10 ml of blood was drawn from the ear vein for the first 7–8 days and then on alternate days for the next 7 days. Five milliliters was used for blood culturing and the rest prepared as serum and stored at −20°C for antibody analysis and serum chemistries. Rectal temperatures were also obtained daily for 7–8 days and then on alternate days. After 14 days the rabbits were sacrificed, and catheters were cultured semiquantitatively by the method of Maki et al. [8]. Experimental protocols were performed using groups of 4–9 rabbits equally divided between PS/A-, TA-, and adjuvant-immunized animals.

Determination of bacteremia. Blood (5 ml) from the ear vein was added to 50 ml of tryptic soy broth (TSB) containing 0.05% polyanetholesulfonic acid (SPS) and incubated for up to 10 days at 37°C. Routine subcultures were made on tryptic soy agar (TSA) and positive growth checked by colonial morphology, Gram's stain, and by flooding the plate with *o*-phenyl phosphate to seek alkaline-phosphatase activity. Strain RP62A does not make alkaline phosphatase, a feature of only 1% of *S. epidermidis* strains [5], whereas strains RP12 and M33 are positive for alkaline phosphatase. Supernates containing bacterial growth were checked by Ouchterlony immunodiffusion analysis [12] to ensure that the PS/A and TA antigens homologous to the infecting strain were made by the isolates recovered from infected blood and catheters. Culture supernates from isolates recovered from rabbits infected with strain M33 were analyzed to be sure that antigens cross-reactive with those from strain RP62A were not elaborated. Strain M33 was also cultured on media containing tetracycline (50 μg/ml), to which it was resistant. Only cultures containing organisms that appeared identical to the infecting strain by these criteria were considered positive.

Immunization of rabbits before infection. Rabbits were immunized subcutaneously with 100 μg of purified PS/A from strain RP62A in complete Freund's adjuvant twice a week for 2–3 weeks. Controls were immunized with either adjuvant alone or with *S. epidermidis* strain SE360.

Passive protection using a dual pump model of catheter-related bacteremia. In this model, we used two catheter-pump combinations in one rabbit. One catheter was infected and implanted into the right jugular as described above. A second sterile catheter was inserted into the left jugular vein. The proximal end of the tubing of the sterile catheter was attached to an osmotic pump filled with 2 ml of either undilute, PS/A-specific polyclonal antibody, 2 ml of monoclonal antibody (2.5 mg/ml), or 2 ml of undiluted normal rabbit serum. Blood cultures were drawn (as described above) daily for 7 days and then on alternate days until day 14, when the rabbits were sacrificed and both infected and noninfected catheters cultured.

Serum chemistries. Metabolic, renal, hepatic, and electrolyte levels were determined on serum samples submitted to the Tufts University School of Veterinary Medicine Diagnostic Laboratories.

Phagocytosis assay. The phagocytosis assay was similar to one previously described [14]. The assay employed *S. epidermidis* strain RP62A, human white blood cells, guinea pig complement, and sera from normal rabbits (NRS) and rabbits immunized with PS/A (IRS). Polymorphonuclear neutrophils (PMNL) and monocytes were prepared from peripheral venous blood and suspended in RPMI with

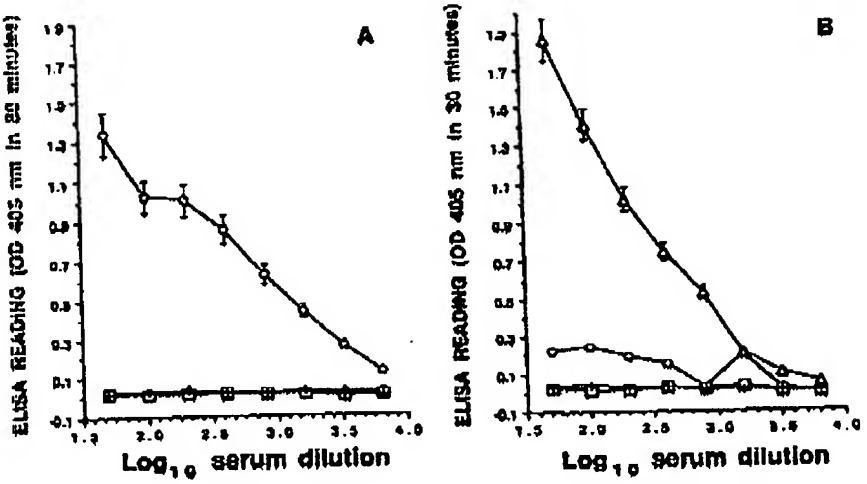

Figure 1. Immune response of rabbits to purified polysaccharide/adhesin (PS/A) (A) or purified teichoic acid (TA) (B) after immunization with either purified PS/A (○, $n = 6$), *S. epidermidis* strain SE360 (TA immune, △, $n = 4$) or complete Freund's adjuvant (□, $n = 8$). Measurements obtained with preimmune serum (+). Points indicate mean of individual serum samples from members of each group; bars indicate standard error of mean.

5% fetal calf serum (FCS). NRS and IRS were heat-inactivated at 56°C for 30 min. PMNL (1×10^6 cells/ml) or monocytes (2×10^6 cells/ml) were incubated at 37°C in RPMI with 5% FCS, guinea pig complement (75 CH_{50} units/ml), diluted rabbit serum (1:4), and *S. epidermidis* strain RP62A (6×10^6 colony-forming units [cfu]/ml) for 2 h. Viable colony-forming units were determined by counting at 30, 60, and 120 min after start of incubation.

Statistical analysis. The method of Connolly and Liang [15] was used to adjust estimates of the protection afforded by active and passive immunization to PS/A. This method was used because of the known interdependence of blood culture results on results from other cultures taken from the same animal. This interdependence violates an assumption of the usual χ^2 test for binomial proportions. The model employs measurements in which the log of the odds of obtaining a positive blood culture on a given day is considered a linear function of the total number of other days for which a positive blood culture is obtained for that animal. Incorporated into this model are terms for the immune status of the animal and the challenge organism (strain RP62A or RP12) employed. Using this method we can determine the degree of intraanimal dependence by estimating the odds ratio (OR) for a positive blood culture for any pair of days from an individual rabbit. This model can also estimate the OR between animals in two groups, taking into account the intraanimal dependence, and thus provide a more conservative statistical analysis on the protective effect of immunization with PS/A.

Differences in glucose and lipid levels were determined using an unpaired *t* test. Differences in daily mean temperature from the preinfection temperature were measured by a paired *t* test.

Results

Description of animal model. In initial experiments to define the parameters of the animal model, we observed the following: Insertion of an infected catheter into the right jugular with heparin flowing through it via an osmotic pump resulted in consistent bacteremia for up to 8 days in normal rabbits. Once the heparin in the osmotic pump was depleted, bacteremia was no longer detectable. Due to this finding we analyzed results from bacteremia for the first 8 days of the experimental period. Quantitative blood cultures in which 1 ml of blood was inoculated into pediatric size (1.5) Isolator culture tubes (Du Pont, Wilmington, DE) and plated onto TSA were positive only in the first 48 h after infection when 1–10 cfu/ml of blood could be detected (not shown). Culturing 5 ml of blood in 50 ml of TSB with 0.05% SPS appeared to be a more sensitive method for detecting bacteremia beyond the first 48 h; thus this technique was used routinely. Additional experiments indicated that no bacteremias were observed if catheters were not infected ($n = 3$ rabbits, 21 blood cultures), if pumps were not filled with heparin dispensed through the catheter ($n = 3$ rabbits, 21 blood cultures), or if infected catheters were inserted into a jugular and removed within 5 min ($n = 2$ rabbits, 14 blood cultures).

Specificity of immunity after immunization with PS/A, S. epidermidis strain SE-360, or adjuvant. Figure 1 depicts the immune response of rabbits to both PS/A and TA after immunization with purified PS/A, *S. epidermidis* strain SE360, or adjuvant. PS/A induced high-titered antibody to itself but not to TA, a major component of unpurified coagulase-negative staphylococcal slime along with PS/A [3]. Strain SE360 induced high-titered antibody to TA but not to PS/A. Purified TA by itself is not immunogenic in rabbits [16] (unpublished data) so a whole bacterial cell inoculum is needed. Strain SE360 has been reported by Ichiman and Yoshida [11] to be encapsulated, but immunization with this strain failed to elicit antibodies to PS/A from strain RP62A. Adjuvant alone also was not immunogenic.

Effect of immunization with PS/A, strain SE360, or adjuvant on bacteremia and catheter colonization by strains RP62A, RP12, and M33. Immunization with PS/A from strain RP62A, but not strain SE360 (TA immune) or adjuvant, reduced the number of positive blood cultures during the first 8 days in

Table 1. Results of daily blood culture for 8 days in immune and nonimmune rabbits implanted with a catheter colonized by *Staphylococcus epidermidis* strain RP62A or strain RP12.

Challenge strain, immune status	No. of animals	Positive cultures/total cultures (%)
RP62A		
Nonimmune*	8	57/64 (89)
PS/A immune	6	14/48 (29)
TA immune†	4	20/32 (62)
RP12		
PS/A immune	3	5/24 (21)
TA immune	3	18/24 (75)

NOTE. PS/A = polysaccharide/adhesin.
* Nonimmune rabbits were immunized with complete Freund's adjuvant.
† TA-immune rabbits were immunized with *S. epidermidis* strain SE360.

animals with catheters infected with either strain RP62A or strain RP12 (table 1, figure 2).

Examination of the results for each animal shown in figure 2 indicated that detection of a positive blood culture in a given animal on a given day could be dependent, in part, on having a prior bacteremia. We therefore used a statistical test of the differences in proportion of bacteremic days among the animals immunized with PS/A, TA, or adjuvant, which adjusted for the degree of dependence observed for the results of blood cultures taken from the same animal [15].

The estimate of the OR for determining intraanimal dependence on the outcome measure of bacteremia was 1.83 (P = .000002; 95% confidence interval [CI], 1.53, 2.19). Thus, as expected, there was strong evidence for the dependence of a positive blood culture being obtained from a rabbit having a prior positive blood culture. Taking this effect into account, the estimate of the OR comparing TA-immune animals to PS/A-immune animals was 1.74 (P = .039; 95% CI, 1.07, 2.85), and the estimate of the OR comparing animals immunized with adjuvant to PS/A immune animals was 2.85 (P = .018; 95% CI, 1.29, 6.30). There was no significant difference in the outcome obtained using either strain RP62A or RP12 as the challenge organism (OR = 0.92, P = .70; 95% CI, 0.59, 1.43), and there was no significant difference in the OR between TA-immune and animals immunized with adjuvant (OR = 0.61, P = .16; 95% CI, 0.31, 1.19). These results indicated a protective efficacy of PS/A immunization but not TA immunization on hematogenous dissemination of coagulase-negative staphylococci from an infected catheter.

In another experiment we challenged immunized rabbits with catheters colonized by *S. epidermidis* strain M93. This strain adheres to silicone elastomer catheters (>300 cfu/catheter from an inoculum of 10^2) as does strain RP62A, but does not produce serologically detectable adhesin by ELISA [7]. In three PS/A and three SE360 (TA) immune rabbits there was no difference in the number of positive blood cultures obtained over 7 days (10 of 21 for PS/A-immune vs. 11 of 21 for TA-immune), although the overall bacteremia rate was lower than that observed in unprotected or TA-immune animals

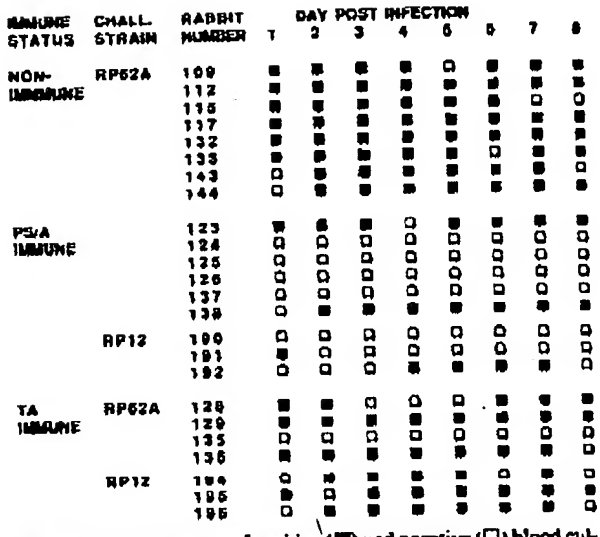

Figure 2. Occurrence of positive (■) and negative (□) blood cultures obtained on postinfection days 1–8 resulting from implantation of catheters infected either with *S. epidermidis* strains RP62A or RP12. PS/A = polysaccharide/adhesin, TA = teichoic acid.

challenged with strains RP62A or RP12 in other experiments (table 1).

Other results from the active immunization experiments. Only occasional blood cultures from these animals were positive after 8 days when the fluid in the pumps was exhausted, indicating that secondary foci of infection were not routinely established. All catheters removed after 14 days of infection were positive for the infecting strain. Most catheters were heavily colonized (>300 cfu/catheter) as determined by the semiquantitative roll-plate technique [8]. Although there was a trend towards catheters from PS/A immune animals to have lower colony counts following semiquantitative culturing, this was not significant. Rabbits in both the immunized and control groups challenged with strains RP62A and RP12 had moderately elevated temperatures 1–5 days after infection. Although rabbits in PS/A immune groups had lower overall mean temperatures after infection, these were not significantly different from animals immunized with adjuvant.

Serum chemistries of infected rabbits. We initially screened selected sera from immune and nonimmune animals for metabolic, renal, hepatic, and electrolyte levels to identify changes associated with bacteremia. We found that bacteremic rabbits were transiently hypoglycemic and hyperlipidemic for several days. Remaining sera obtained every 2–3 days during the observation period were then analyzed for glucose and lipid levels. Glucose levels were significantly (P < .01) depressed in rabbits immunized with TA and adjuvant compared with PS/A immune rabbits 5, 7, and 10 days after infection, indicating that this effect was present even after bacteremia had

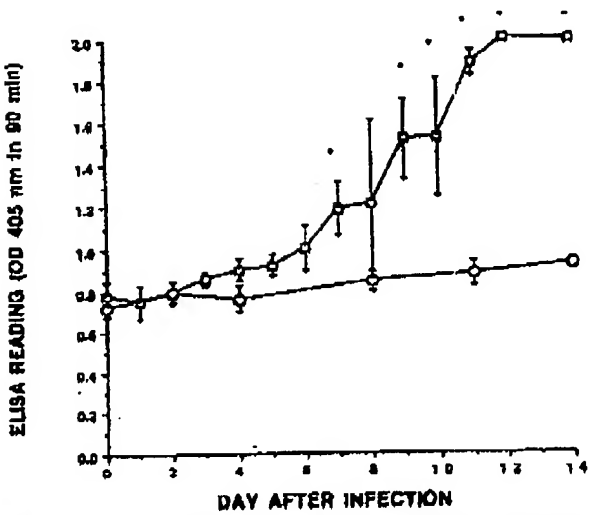

Figure 3. Immune response to teichoic acid (TA) in rabbits implanted with catheters infected with *S. epidermidis* strain RP62A and immunized with either polysaccharide/adhesin (PS/A) (○, n = 6) or complete Freund's adjuvant (□, n = 8). Sera from each day were diluted 1:10 for testing in triplicate. Points represent mean of individual serum samples for each group and bars represent standard error of mean. * = significant ($P < .05$, unpaired t test) difference from day 0 serum sample. Level seen in PS/A immune rabbits is higher than observed in figure 1 due to longer incubation time of ELISA plate and higher serum concentration tested.

ceased. Triglycerides were elevated between 3 and 10 days after the onset of bacteremia in the rabbits immunized with TA and adjuvant. These assays provided an independent measure of the effect of PS/A immunization on protection against bacteremia.

Immune response of rabbits during infection with strain RP62A. We compared the immune response of rabbits immunized with PS/A, TA, and adjuvant to PS/A and TA during the 14 days after challenge. Rabbits immunized with adjuvant and infected with strain RP62A made antibody to TA (figure 3) but not to PS/A (not shown) during the experimental period. In contrast, rabbits immunized with PS/A and challenged with either strains RP62A or RP12 did not produce antibodies to TA (figure 3) and did not have a further increase in their PS/A-specific titers (not shown).

Rabbits immunized with TA and challenged with either strain failed to respond to infection with antibody to PS/A within 14 days and had no further rise in antibody titer to TA (not shown). However, TA-immune rabbits challenged with strain RP12 showed a two- to fourfold increase 10–14 days after infection by ELISA reading to whole cells of strain RP12 (not shown). PS/A-immune animals challenged with strain RP12 showed no change in ELISA reading to whole cells. For animals challenged with strain M33, both PS/A- and TA-immune rabbits experienced a two- to fourfold increase in ELISA reading to whole cells of strain M33 and neither group showed changes >20% in the ELISA reading to PS/A or TA from strain RP62A.

Table 2. Results of daily blood culture for 7 days in rabbits implanted with a catheter colonized by *Staphylococcus epidermidis* strain RP62A and passively infused with either normal serum or polyclonal or monoclonal antibody to polysaccharide/adhesin (PS/A).

Passive infusion	No. of animals	Positive cultures/ total cultures (%)
Normal serum	5	28/35 (80)
PS/A immune serum*	6	9/42 (21)
PS/A MAb†	6	13/42 (31)

* PS/A immune serum obtained from rabbits immunized with purified PS/A.
† PS/A MAb was a murine monoclonal antibody specific for PS/A.

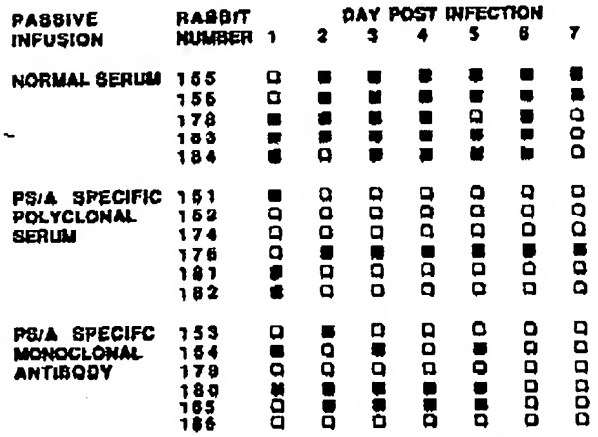

Figure 4. Occurrence of positive (■) and negative (□) blood cultures obtained on days 1-7 after infection resulting from implantation of catheters infected with *S. epidermidis* strain RP62A in rabbits passively infused with either normal serum or polyclonal or monoclonal antibodies specific for strain RP62A polysaccharide/adhesin (PS/A).

Passive protection against strain RP62A infection using polyclonal and monoclonal antibody to homologous PS/A. In this model, infusion of either polyclonal immune serum raised to purified PS/A, or a monoclonal antibody to PS/A, reduced the number of positive blood cultures during an experimental period of 7 days compared with rabbits passively infused with normal serum (table 2, figure 4). Due to technical difficulties in obtaining sufficient blood from some rabbits, day 8 samples were not available from all animals and hence were not included in the data analysis. Analysis of these data by the method of Connolly and Liang [15] again indicated a strong dependence among blood cultures obtained from the same animal (OR = 1.58, P = .005; 95% CI, 1.21, 2.05). Comparison of the blood culture results in animals receiving

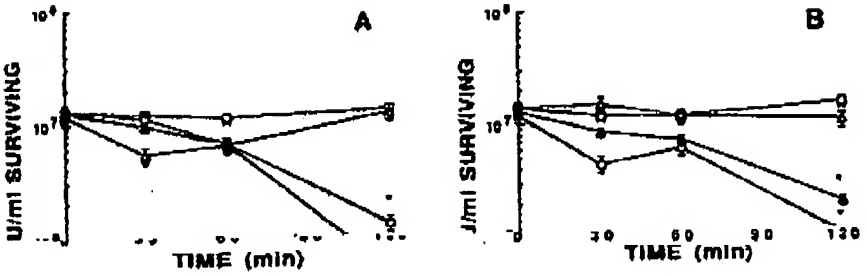

Figure 5. Killing of *S. epidermidis* strain RP62A over 120 min by either human polymorphonuclear neutrophils (A) or mononuclear cells (B) in the presence of guinea pig complement and normal rabbit serum (A), rabbit serum raised to polysaccharide/adhesin (PS/A) from strain RP62A (O), monoclonal an-

normal serum infusions with those receiving infusions of PS/A-specific polyclonal antisera showed a significant difference (OR = 3.39, $P = .029$; 95% CI, 1.27, 9.02) as did comparison of the blood culture results of animals infused with normal serum with those receiving PS/A-specific monoclonal antibody (OR = 2.68, $P = .041$; 95% CI, 1.14, 6.31).

Culture of the initially noninfected catheter after the experimental period showed all five catheters from rabbits receiving normal serum were infected, whereas 6 of 10 catheters from rabbits receiving the immunotherapies were sterile ($P \leq .05$, Fisher's exact test). Since the number of colony-forming units per infected catheter were comparable regardless of the passive therapy employed, there was no significant difference in the quantitative level of organisms colonizing the catheters. Glucose levels were depressed in rabbits receiving normal serum 6–9 days after infection, and triglyceride levels rose in rabbits receiving normal serum compared with those receiving passive immunotherapies between days 3 and 11 after the onset of bacteremias.

Phagocytosis results. Figure 5 shows the killing over 120 min of *S. epidermidis* strain RP62A by antisera and monoclonal antibody to PS/A compared with normal rabbit serum and no serum (tissue culture medium substituted) in the presence of human PMNL or mononuclear phagocytes and guinea pig complement. In both instances, immune serum and the monoclonal antibody was significantly ($P < .001$) more opsonic than normal serum at the 120-min sampling point.

Discussion

Our previous studies have demonstrated the role of the capsular PS/A of coagulase-negative staphylococci in mediating attachment to foreign bodies and the ability of antibody directed against PS/A to block this in vitro adherence [6]. Since some strains of coagulase-negative staphylococci appear to be encapsulated [11, 17], it is important to consider the possibility that capsular PS/A might have a more traditional function typical of other encapsulated pathogens. Capsular polysaccharides of many bacterial pathogens help to protect these organisms against host defenses, principally by interfering with phagocytosis. Not surprisingly, specific antibody to the capsular antigens of these bacteria prevents invasive disease. The studies reported here suggest that in addition to promoting adherence to foreign bodies, PS/A also may protect coagulase-negative staphylococci against phagocytosis and antibody to PS/A may neutralize this shield and impede invasion of the bloodstream by organisms colonizing intravascular catheters. We demonstrated that antibody to PS/A is opsonophagocytic and that immunization with PS/A-protected rabbits against bacteremia with the homologous strain RP62A and the heterologous (but serologically identical) strain RP12. Passive immunotherapy with polyclonal and monoclonal antibodies infused through an uninfected catheter also reduced bacteremia spawned by the contralateral contaminated catheter.

In addition to limiting catheter-associated bacteremia, both active and passive immunotherapy attenuated the changes in serum levels of glucose and triglycerides associated with bacteremia in the rabbit. Consistent development of hypoglycemia and hyperlipidemia were seen in unprotected animals. Based on subjective observations of the animals, the hypoglycemia did not appear to be due to decreased food intake or defecation. We did not quantitate food intake or fecal production so this observation is speculative. Hyperlipidemia is a pronounced feature of rabbits receiving infusions of toxic shock syndrome toxin-1 [18] through an osmotic pump and may represent a common response of rabbits to stress.

We found strong immune responses to TA, but not to PS/A, within the first 14 days after catheter implantation and development of bacteremia in unimmunized animals. Thus PS/A may be poorly immunogenic during infection, while nonprotective antibodies to TA are readily elicited. Alternatively, the observation period of 14 days may have been too short to observe development of antibody to PS/A. We have found,

however, that immunization of rabbits and mice with purified PS/A elicits much higher antibody titers than immunization with whole bacterial cells (unpublished data).

One measure of infection that did not differ among the various groups was fever. All groups of animals showed a modest rise in mean temperature that was significantly different for up to 5 days from the preinfection level. It is not clear if temperature elevation was a measure of infection, suggesting that PS/A-immune animals were bacteremic but at a level below that detectable by our blood culturing technique, or if this was a response to the surgery and subsequent stress experienced by all animals. We believe the latter may be the case since temperature measurements in rabbits following surgery to implant sterile catheters for other experimental purposes were also elevated to a comparable degree (unpublished data).

Although passive immunization against PS/A protected against hematogenous seeding and colonization of noncontaminated catheters, we were unable to determine if this was due to an antiadhesive property of PS/A-specific antibody [6] or to the opsonophagocytic effect of this antibody on bloodborne coagulase-negative staphylococci. The failure of antibody to sterilize contaminated catheters is not surprising since these catheters were exposed to high concentrations of coagulase-negative staphylococci before insertion. Thus, the PS/A adhesin may have located its target on the catheter before adherence could be interrupted by antibody to PS/A. Further studies will be needed to ascertain whether preexisting antibody can prevent catheter colonization in circumstances that more closely resemble the clinical situation in which the catheter is contaminated by small numbers of organisms from the skin of the patient during or after insertion.

Our results demonstrate a role for PS/A-specific antibodies in protecting rabbits against bacteremia and possibly against catheter colonization in this model. Preliminary analysis (unpublished data) of antibodies to this antigen in normal human sera indicates a high prevalence of naturally occurring antibody to PS/A. If antibody to PS/A is important in protective immunity against coagulase-negative staphylococcal infection, this would suggest that invasive infection may occur more frequently in individuals with decreased or nonfunctional (i.e., nonopsonic) antibodies to PS/A. We are currently investigating this in acute phase serum samples obtained from infected patients. Overall our findings in this animal model suggest that immunotherapies directed at PS/A could be protective against coagulase-negative staphylococcal bacteremia.

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Appendix C

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Protection against endocarditis due to Staphylococcus epidermidis by immunization with capsular polysaccharide/adhesin

S Takeda, GB Pier, Y Kojima, M Tojo, E Muller, T Tosteson and DA Goldmann

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Protection Against Endocarditis Due to *Staphylococcus epidermidis* by Immunization With Capsular Polysaccharide/Adhesin

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Background. ***Staphylococcus epidermidis*** **is the principal pathogen in prosthetic valve endocarditis. The capsular polysaccharide adhesin (PS/A) has been shown to mediate attachment of bacteria to medical devices. In this study, we investigated the efficacy of active and passive immunization against PS/A in preventing *S. epidermidis* endocarditis in a rabbit model.**

Methods and Results. **Aortic valve vegetations were produced by inserting a Teflon catheter into the left ventricle through the right carotid artery. Bacteremia and endocarditis were then established by implanting in the left jugular vein a catheter that was attached to an osmotic pump and contaminated with *S. epidermidis* strain RP62A. During a 3-week study period, of 64 blood cultures taken every second or third day from six nonimmune rabbits, 54 (84%) yielded strain RP62A. In rabbits actively immunized with PS/A, eight of 60 blood cultures (13%) were positive (odds ratio 5.0, 95% CI, 2.0–12.3, $p=0.005$). At death, all six nonimmune rabbits had infected vegetations that yielded 10^8–10^{11} colony-forming units (cfu)/g of vegetation, whereas only one PS/A-immunized rabbit had an infected vegetation. Immunization protocols designed to elicit antibody to teichoic acid but not to PS/A afforded no protection against bacteremia or endocarditis. Infusion of monoclonal antibody to PS/A through a catheter in the right jugular vein provided a level of protection against both bacteremia and endocarditis comparable to that produced by active immunization. In vitro, antibody against PS/A was opsonic for *S. epidermidis*.**

Conclusions. **Immunoprophylaxis targeted at staphylococcal PS/A is a promising new approach to the prevention of prosthetic valve endocarditis. (*Circulation* 1991;84:2539–2546)**

The coagulase-negative staphylococcus, particularly *Staphylococcus epidermidis*, is a formidable pathogen in patients undergoing prosthetic cardiac valve surgery. Not only is *S. epidermidis* the principal pathogen in prosthetic valve endocarditis,[1–8] but it is also the most frequent cause of intravascular catheter-associated infections in the immediate postoperative period.[9–12] Because *S. epidermidis* is a major component of the normal skin flora and very rarely causes infection in the absence of a foreign body, a number of investigators have hypothesized that this microorganism has a special ability to adhere to, colonize, and infect prosthetic materials because of its unique surface properties. Attention has focused on the ability of some strains, particularly those isolated from infections of medical devices, to produce copious amounts of an extracellular material generally referred to as "slime."[9,13–21] Some investigators believe that slime mediates adherence of staphylococci to prosthetic materials. In addition, it has been noted that slime envelops adherent bacterial colonies in a thick biofilm that may provide protection from host defenses and antibiotics. Despite considerable investigation, however, the precise role of this complex material in the pathogenesis of device-associated coagulase-negative staphylococcal infections remains unclear.

We have previously purified a polysaccharide from slime-producing strains of *S. epidermidis* that appears to be involved in staphylococcal adherence to plastics, such as silicon elastomer, that are frequently used in catheters and other medical devices.[22] In addition to functioning as an adhesin, this polysac-

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charide also serves as a capsule for *S. epidermidis*. Most clinical isolates of *S. epidermidis* were found to produce a serologically identical capsular polysaccharide adhesin (PS/A). Chemically, PS/A is a large (more than 500,000 kd) polymer of neutral sugars rich in galactose and arabinose. In vitro, antibody to purified PS/A inhibited adherence of a number of strains of *S. epidermidis* to silicon elastomer tubing, which suggests that immunoglobulin directed specifically against PS/A might have a role in the prevention of coagulase-negative staphylococcal infections by inhibiting bacterial attachment.

We then extended these in vitro findings to a rabbit model of central venous catheter infection, demonstrating that active immunization with PS/A greatly attenuated bacteremia spawned by catheters that had been contaminated with *S. epidermidis*.[23] Passive infusion of PS/A-specific polyclonal and monoclonal immunoglobulin through a separate catheter placed in the contralateral jugular vein protected the rabbits against both bacteremia and hematogenous colonization of this contralateral catheter. In the present study, we have further explored the potential efficacy of immunoprophylaxis in a rabbit model of endocarditis

Methods

Bacterial Strains

S. epidermidis strain RP62A is a previously described[19,22] prototype slime-producing clinical isolate from which we originally extracted and purified PS/A. *S. epidermidis* strain SE360[23,24] expresses teichoic acid (TA) and several surface proteins serologically identical to those of strain RP62A but does not elicit antibody to PS/A in rabbits.

Antibody Assays and Production of Polyclonal and Monoclonal Antibodies to PS/A

Capsular PS/A was purified as described previously.[22] To obtain polyclonal antibodies, rabbits were immunized twice weekly for 3 weeks with subcutaneous injections of 100 μg PS/A from strain RP62A in complete Freund's adjuvant. Antisera were tested for antibody to PS/A and TA by enzyme-linked immunosorbent assay (ELISA) as described previously.[22] Postimmunization sera were tested at dilutions of 1:100 to 1:51,200 and compared to titers in sera obtained just before immunization; postinfection sera were tested at a dilution of 1:100 only, and ELISA readings obtained at this dilution were compared with those obtained using a 1:100 dilution of prechallenge sera. Individual sera were tested in duplicate, and means for groups of similarly treated rabbits represent the means derived from the means of the individual duplicate titers.

Monoclonal antibody was prepared by standard techniques[25] after hyperimmunization of BALB/c mice with 10 μg purified PS/A. One PS/A-specific clone producing an IgG3 subclass antibody and designated IXB2 was used in passive immunoprophylaxis studies. This antibody was obtained from culture supernates of hybridoma cells producing IXB2 antibody after application to a Bakerbond ABx column (J.T. Baker, Phillipsburg, N.J.) and elution with 1 M NaCl and 0.5 M ammonium sulfate. The purified antibody was then dialyzed against phosphate buffered saline.

Rabbit Model of Catheter-Induced Endocarditis

New Zealand White rabbits weighing 2.5–3.0 kg were anesthetized with ketamine (Ketalar, 40 mg/kg, Parke-Davis, Morris Plains, N.J.), atropine (100 μg), and xylazine (Rompun, 10 mg/kg, Mobay, Shawnee, Kan.). The neck area was shaved and disinfected with iodine tincture, and a 3-cm incision was made to expose the right carotid artery. A 3F Teflon catheter was inserted into the carotid by cutdown, and the catheter was then passed through the carotid artery into the cavity of the left ventricle (Figure 1). The incision was closed after the tubing was tied in place. This procedure resulted in production of aortic valve leaflet vegetations in all rabbits within 7 days. One week after catheter insertion, rabbits were challenged by insertion of a 2.7F silicon elastomer catheter contaminated by immersion in a suspension of 10^8 cfu/ml of strain RP62A for 15 minutes. After anesthesia and skin disinfection, a 3-cm incision was made to expose the left jugular vein, and the catheter was inserted 4–5 cm into the vein by cutdown. The external end of the catheter was attached to a subcutaneous osmotic pump (Alzet model 2ML1, Alza Corp., Palo Alto, Calif.) (Figure 1) The pump was filled with 2 ml of 10,000 units/ml heparin, which was delivered continuously for 7 days at 10 μl/hr. This technique had previously been found to result in continuous low-grade bacteremia (less than 10 cfu/ml of blood[23]) for up to 8 days in normal rabbits. Once the heparin in the pump was depleted, bacteremia was no longer detectable. In the endocarditis model, however, sustained bacteremia beyond 8 days was noted, indicating the formation of an independent focus of infection (see below).

Immunoprophylaxis Regimens

For active immunization experiments, rabbits were immunized subcutaneously with 100 μg of purified PS/A from strain RP62A in complete Freund's adjuvant twice a week for 2–3 weeks. This immunization schedule was sufficient to produce a significant increase in anti-PS/A antibody titers by ELISA in all rabbits. Control rabbits were immunized with either adjuvant alone or whole cells of *S. epidermidis* strain SE360, which we have previously shown[23] elicits high titers of antibody to cell wall TA but does not elicit antibody to the PS/A of strain RP62A.

For passive immunotherapy, a dual pump rabbit endocarditis model was used to evaluate the protective efficacy of monoclonal antibody (Figure 1). In these experiments, a second silicon elastomer catheter, which had not been contaminated with *S. epidermidis*, was inserted into the right jugular vein and attached to an osmotic pump filled with 2 ml of either normal rabbit serum or monoclonal antibody IXB2.

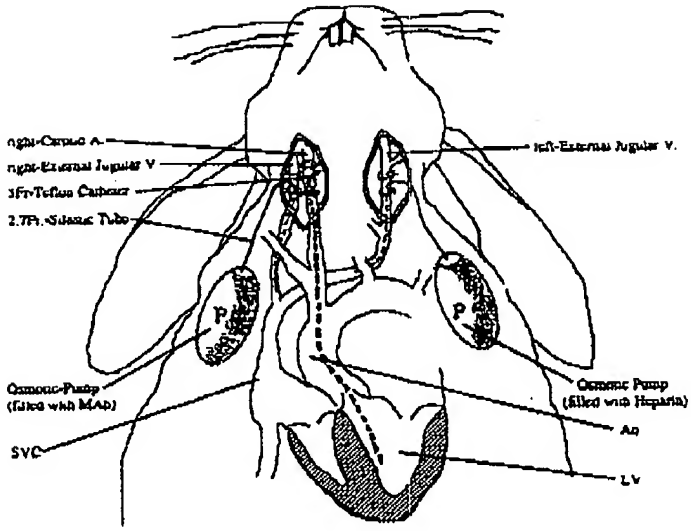

FIGURE 1 *Schematic diagram of the rabbit model of endocarditis used in these studies showing the positions of the catheter inserted through the right carotid artery (right-Carotid A.) to induce aortic valve lesions; the contaminated catheter implanted into the left external jugular vein (left-External Jugular V.) and attached to an osmotic pump; and the sterile catheter in the right external jugular vein (right-External Jugular V.) used to infuse monoclonal antibody or normal serum in passive immunotherapy experiments. Ao, aorta; LV, left ventricle; SVC, superior vena cava; MAb, monoclonal antibody.*

Determination of Bacteremia and Endocarditis

Blood cultures were performed just before challenge and every 2–3 days thereafter until the animals were killed. Blood (5 ml) from the ear vein was added to 50 ml of tryptic soy broth containing 0.05% polyanetholsulfonic acid and incubated for up to 10 days at 37°C. Routine subcultures were made on tryptic soy agar. All blood culture isolates were verified as the challenge strain RP62A by colonial morphology, Gram's stain, and biochemical testing. Identification of blood isolates was facilitated by the fact that strain RP62A is alkaline phosphatase–negative.[23] Supernates of positive cultures were checked by Ouchterlony immunodiffusion[26] to ensure that PS/A and TA antigens homologous to the infecting strain were made by the bloodstream isolates.

Twenty to 22 days after bacterial challenge, the rabbits were killed, and cardiac vegetations, the intraventricular Teflon catheter, and the intrajugular silicon elastomer catheters were examined and removed. Rabbits that died before the end of the experimental period were processed immediately in the same way. Vegetations were weighed, homogenized, diluted in saline, and cultured quantitatively on tryptic soy agar. Results were expressed as log cfu/g of vegetation. Catheters were cultured semiquantitatively by rolling a 1-cm distal segment on tryptic soy agar plates as described by Maki et al.[27] Isolates of *S. epidermidis* were verified as strain RP62A as described above.

Serum Chemistries

Glucose and lipid levels were determined on serum samples submitted to the Tufts University School of Veterinary Medicine Diagnostic Laboratories.

Opsonophagocytosis Assay

Opsonophagocytosis was measured by an assay similar to one described previously.[28] The assay incorporated *S. epidermidis* strain RP62A, human polymorphonuclear leukocytes, rabbit complement adsorbed with 10^9 cfu/ml of strain RP62A, and serum from either normal rabbits, rabbits immunized with PS/A, or the monoclonal antibody 1XB2. White blood cells were prepared from human peripheral venous blood and overlaid on mono/poly resolving medium (Flow Laboratories, Inc., McLean, Va.). Rabbit sera were heat-inactivated at 56°C for 30 minutes. Purified (more than 95%) polymorphonuclear leukocytes (1×10^6 cells/ml) were suspended in RPMI with 5% fetal calf serum and incubated at 37°C for 90 minutes with adsorbed rabbit complement (1:20 dilution), rabbit serum (1:10 dilution) or monoclonal antibody (2.5 μg/ml), and strain RP62A (6×10^6 cfu/ml). Viable cfus were determined by counting at the end of this incubation period.

Statistical Analysis

The method of Connolly and Liang[29] was used to adjust estimates of the protection afforded by active and passive immunization. This method was used because of the known interdependence of blood culture results and results from other cultures taken from the same animal. This interdependence violates an assumption of the usual χ^2 test for binomial proportions. The model uses measurements in which the log of the odds of obtaining a positive blood culture on a given day is considered a linear function of the total number of other days for which a positive blood culture is obtained for that animal. Incorporated into this model are terms for the immune status of the animal and the challenge organism used. With this method we can determine the degree of intra-animal dependence by estimating the odds ratio for a positive blood culture for any pair of days from an individual rabbit. This model can also estimate the odds ratio between animals in two groups, taking into account the intra-animal dependence, and thus pro-

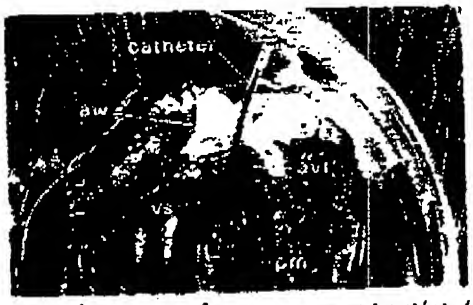

FIGURE 2. *Photograph of vegetation on aortic valve of rabbit challenged with* Staphylococcus epidermidis *RP62A. aw, Aortic wall; avl, aortic valve leaflets with vegetations; vs, ventricular septum, pm, papillary muscle.*

vide a more conservative statistical analysis on the protective effect of immunization with PS/A.

Differences in antibody levels between the preinfection and postinfection sera were measured by a paired *t* test. Opsonophagocytic killing was compared by a Student's *t* test.

Results

Bacteremia and Endocarditis in Rabbits Challenged With S. epidermidis *Strain RP62A*

All rabbits in these experiments developed *S epidermidis* endocarditis after challenge with the contaminated catheter unless they were protected by active or passive immunization against PS/A. Large infected vegetations were noted uniformly on the aortic valve of nonimmune rabbits (Figure 2), with concentrations of *S epidermidis* ranging from 10^8 to 10^{11} cfu/g of vegetation (Figures 4 and 6). Sustained bacteremia occurred in all nonimmune rabbits (Figures 3 and 5). All Teflon catheters inserted through the aortic valve into the left ventricle were found to be coated with a fibrinous sleeve, with 20 to more than 1,000 colonies noted on semiquantitative catheter cultures. Culture-positive vegetations formed on the left ventricular wall opposite the tip of the Teflon catheter in some rabbits, particularly when the catheter tip and myocardium were closely apposed; these vegetations were not cultured quantitatively. Semiquantitative cultures of the contaminated silicon elastomer jugular catheters were still positive when they were removed from the rabbits at the end of the experiments, yielding 380 to more than 1,000 colonies per plate.

Several variations on this experimental protocol were used to determine the parameters necessary for establishing endocarditis. When three rabbits with catheters inserted into the left ventricle were challenged with contaminated jugular catheters attached to osmotic pumps that did not contain heparin, bacteremia and endocarditis did not develop, probably because of a lack of fluid flow through the catheter. Removal of the left ventricular catheter before implantation of the infected catheter–pump combination failed to produce endocarditis, although bacteremia for up to 7 days was noted ($n=3$). When rabbits with left ventricular catheter-induced valvular vegetations were infected with a bolus dose of 10^6 cfu/rabbit of *S. epidermidis* strain RP62A, endocarditis was established, but immune rabbits were not protected. Presumably this very high bacterial inoculum overwhelmed host defenses. Finally, three rabbits with left ventricular catheters that received a sterile catheter–pump combination 1 week later failed to develop bacteremia or endocarditis over the next 3 weeks.

Bacteremia and Endocarditis in Actively Immunized Rabbits

Similar to results previously reported,[24] immunization of rabbits with PS/A from strain RP62A induced

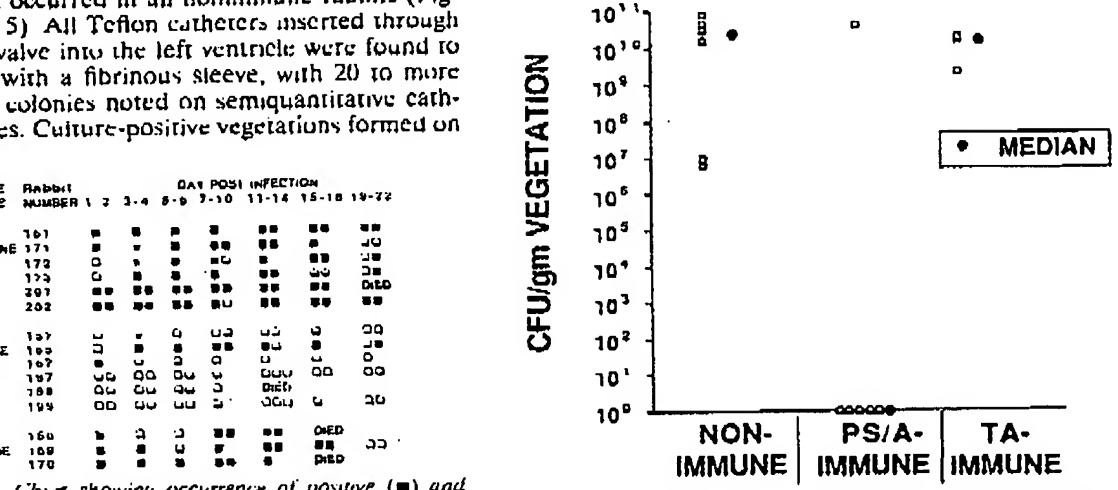

FIGURE 3. *Chart showing occurrence of positive (■) and negative (□) blood cultures obtained on postinfection days 1–22 resulting from insertion of catheters contaminated with* Staphylococcus epidermidis *strain RP62A. Rabbits were either immunized with adjuvant only (nonimmune), immunized with purified PS/A (PS/A immune), or immunized with* S. epidermidis *strain SE360 (TA immune). ▲, No culture taken. PS/A, polysaccharide adhesin; TA, teichoic acid.*

FIGURE 4. *Graph showing concentration (cfu/g) of* Staphylococcus epidermidis *RP62 in aortic valve vegetations of nonimmune rabbits and rabbits immunized with either purified PS/A or* S. epidermidis *strain SE360 (TA-immune). The open boxes (□) indicate values for individual rabbits, and the closed circles (●) indicate the median for each group. PS/A, polysaccharide adhesin; TA, teichoic acid.*

TABLE 1. Summary of Blood Culture Results, Actively Immunized Rabbits

Immune status	No of rabbits	Positive blood cultures/total cultures	Percent positive
Nonimmune*	6	54/64	84
PS/A-immune†	6	8/60	13
TA-immune‡	3	18/23	78

*Non-immune rabbits immunized with complete Freund's adjuvant

†PS/A-immune rabbits immunized with purified polysaccharide adhesin from *S. epidermidis* strain RP62A.

‡TA-immune, teichoic acid–immune rabbits immunized with *S epidermidis* strain SE360.

a high-titer antibody response to PS/A but not to TA, whereas immunization with *S. epidermidis* strain SE360 resulted in the production of antibody to TA but not to PS/A (not shown). There was a marked reduction in the percentage of blood cultures that were positive for *S epidermidis* strain RP62A during the 20–22-day study period in PS/A-immunized rabbits. Sustained bacteremia was noted in only one of six PS/A-immune rabbits versus nine of nine rabbits in the nonimmune and strain SE360-immune (TA-immune) groups ($p<0.01$, Fisher's exact test, Figure 3). Only 13% of all blood cultures were positive in the PS/A-immune rabbits, compared with 84% and 78% in the nonimmune and TA-immune groups, respectively (Table 1). The reason for the death of rabbit 198 could not be ascertained at autopsy.

Examination of the results in Figure 3 indicated that detection of a positive blood culture on a given day could be dependent, in part, on having a previous positive blood culture. Using the method of Connolly and Liang[29] to take this effect into account, the estimate of the odds ratio for obtaining a positive blood culture comparing TA-immune rabbits with PS/A-immune rabbits was 7.69 (95% CI, 2.8–20.8, $p=0.002$). The estimate of the odds ratio for obtaining a positive blood culture comparing nonimmune and PS/A-immune rabbits was 5.00 (95% CI, 2.0–12.3, $p=0.005$).

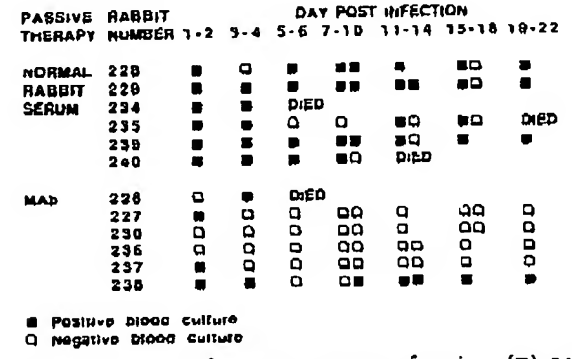

FIGURE 5. *Chart showing occurrence of positive (■) and negative (□) blood cultures obtained on postinfection days 1–22 resulting from insertion of catheters contaminated with* Staphylococcus epidermidis *strain RP62A. Rabbits were passively infused by an osmotic pump attached to a catheter inserted into the right jugular vein containing either normal rabbit serum or monoclonal antibody to PS/A (MAb).*

Only one of six rabbits immunized with PS/A had aortic valve vegetations that yielded *S. epidermidis* on culture (Figure 4). This one rabbit, which also had sustained bacteremia (Figure 3), had 4.0×10^{10} cfu/g of vegetation, and 25 colonies of *S. epidermidis* were recovered on semiquantitative culture of the left ventricular catheter. In contrast, vegetations obtained from nonimmune and TA-immune rabbits were all infected with very high concentrations of bacteria (Figure 4), and all left ventricular catheters were culture-positive as well.

Contaminated jugular catheters used to challenge the rabbits with *S. epidermidis* remained culture-positive in all experimental groups, although the number of colonies recovered from these catheters was slightly lower in PS/A-immune rabbits (3.8×10^1 to 6.1×10^2 cfu/catheter versus 3.6×10^2 to more than 10^3 in the nonimmune and 1.3×10^2 to 8.4×10^3 cfu/catheter in the TA-immune group).

Bacteremia and Endocarditis in Rabbits Passively Immunized With Monoclonal Antibody to PS/A

Passive infusion of monoclonal antibody to PS/A in the dual pump endocarditis model significantly attenuated bacteremia compared with unprotected rabbits (Figure 5, Table 2). All rabbits receiving normal rabbit serum had sustained bacteremia, and 77% of

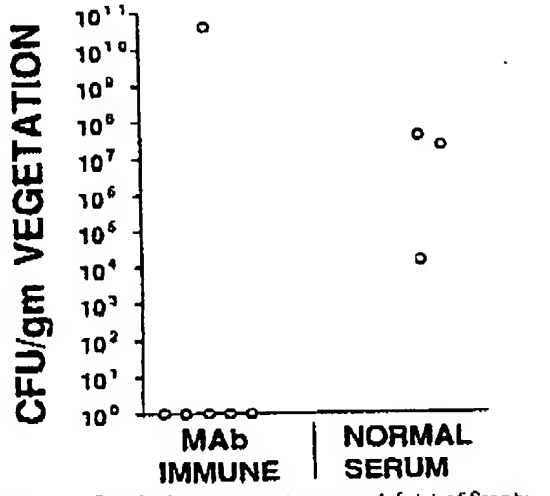

FIGURE 6. *Graph showing concentration (cfu/g) of* Staphylococcus epidermidis *RP62A in aortic valve vegetations of rabbits passively infused with either normal rabbit serum or monoclonal antibody to PS/A (MAb immune). Quantitative values for the three rabbits infused with normal serum that died (see Figure 5) were not obtained, but these vegetations were all found to be colonized with* S. epidermidis *strain RP62A by culturing the vegetations in tryptic soy broth. All five of the vegetations from rabbits infused with the MAb were sterile by this method.*

Table 2. Summary of Blood Culture Results, Rabbits Receiving Passive Immunoglobulin Prophylaxis

Immune therapy infused	No. of rabbits	Positive blood cultures/total cultures	Percent positive
Normal serum	6	43/43	79
MAb to PS/A*	6	10/47	21

*Rabbits infused with monoclonal antibody (MAb) IXB2, specific for capsular polysaccharide adhesin (PS/A) of *S. epidermidis* strain RP62A

all blood cultures obtained in the 20–22-day experiment were positive. One of six passively immunized rabbits had sustained bacteremia (although one other rabbit was bacteremic at the time of death on day 3), and 21% of all blood cultures were positive. The odds ratio for obtaining a positive blood culture from a control rabbit versus immunized rabbits was 5.5 (95% CI, 1.6–18.9, $p=0.02$).

One of the six rabbits given monoclonal antibody had infected aortic valve vegetations (Figure 6); the remaining five rabbits were culture-negative even when nonquantitative broth cultures were used. Rabbits given normal serum infusions were all culture-positive; in three of these rabbits quantitative cultures of vegetations were not obtained, but broth cultures were positive for *S. epidermidis* strain RP62A. Catheters were not routinely cultured in these experiments.

Other Measures of Infection

As in results previously reported for rabbits with catheter-related bacteremia caused by *S. epidermidis*,[23] the rabbits infected here also developed hypoglycemia and hyperlipidemia concurrent with positive blood cultures. These metabolic changes were noted for infected rabbits throughout the entire 3-week study period and provide an independent confirmation of infection in the rabbits. PS/A immunized or monoclonal antibody–infused rabbits did not develop hypoglycemia or hyperlipidemia unless they also had recurring positive blood cultures.

We have also previously reported that rabbits infected with *S. epidermidis* make antibodies to the TA antigen as a result of infection. Similar findings were seen in the rabbits that developed endocarditis, in that rabbits actively immunized with adjuvant or infused with normal serum made antibody to TA isolated from strain RP62A (not shown). Significant ($p<0.05$) increases in titers were observed from 7 days postinfection to the end of the experimental period. Rabbits actively immunized with PS/A or passively infused with PS/A-specific monoclonal antibody did not develop endocarditis or have a significant increase in antibody to TA. Two exceptions were rabbits 165 and 238, which had received an otherwise protective immunotherapy but nonetheless developed bacteremia and endocarditis. These rabbits made immune responses to TA. Rabbits immunized with *S. epidermidis* strain SE360 had preexisting high levels of antibody to TA and made no further immune response to this antigen after infection (not shown).

In previous studies, rabbits with catheter-related coagulase-negative staphylococcal bacteremia failed to make antibody to PS/A as a result of infection.[23] We again found that rabbits actively immunized with adjuvant or *S. epidermidis* strain SE360 failed to make antibody to PS/A (not shown), despite the development of bacteremia and endocarditis (Figures 3 and 4). In the passive protection study, however, rabbits infused with normal serum made a modest but significant ($p<0.05$) immune response to PS/A starting 12 days after infection. The reason for this is not clear; this represents the only situation where we have found an immune response to PS/A among infected rabbits.[23] Rabbits infused with monoclonal antibody to PS/A, including the infected rabbit, 238, failed to make antibody to PS/A during the 3-week experimental period.

Opsonophagocytic Assays

Killing of *S. epidermidis* strain RP62A by human polymorphonuclear leukocytes was observed after 90 minutes in the presence of both monoclonal and polyclonal antibody to PS/A from strain RP62A. Comparable polyclonal antibody preparations have been tested previously and found to mediate opsonic killing,[23] while the monoclonal antibody IXB2 used in passive therapy of endocarditis has not been tested previously in opsonophagocytosis assays. The monoclonal antibody at a concentration 1/10 of that used for passive protection against endocarditis was significantly more opsonic than normal serum, mediating phagocytic killing of 70.7% ($p<0.001$) of the inoculum by 90 minutes (not shown). This was comparable to the level achieved by a 1:10 dilution of PS/A-immune polyclonal rabbit serum (65.9% killed). In contrast, in normal rabbit serum the inocula grew to 171% of the initial bacterial concentration during the incubation period.

Discussion

The results of the present study suggest that immunoprophylaxis may provide a reasonable approach to the problem of preventing endocarditis caused by coagulase-negative staphylococci. We have demonstrated that immunization with a surface polysaccharide of *S. epidermidis* prevents bacterial endocarditis in a rabbit model. Passive infusion of monoclonal antibody to this polysaccharide was equally effective. The precise mechanism for antibody-mediated protection remains to be resolved. It is tempting to speculate that antibody to the polysaccharide, whether actively or passively acquired, interrupts the first step in the pathogenesis of infection—attachment of the staphylococcus to the foreign body. Indeed, monoclonal antibody inhibits adherence of *S. epidermidis* to silicon elastomer and other plastics in vitro (unpublished data). Although we have not tested the role of staphylococcal PS/A in mediating adherence to all of the specific materials used in

prosthetic valve surgery, considerable clinical experience suggests that *S. epidermidis* avidly colonizes and infects a wide range of prosthetic materials of various compositions.[10,13,15,30–32]

On the other hand, it is possible that antibody to *S. epidermidis* polysaccharide protects against infection by a more conventional immune mechanism, opsonophagocytosis of bacteria in blood. In addition to functioning as an adhesin, PS/A is an important component of the capsule of *S. epidermidis*. We found that antibody to PS/A promoted opsonophagocytic killing of *S. epidermidis* in the presence of polymorphonuclear leukocytes, as would be expected for an antibody raised against a bacterial capsular antigen. We have found serologically identical polysaccharide on the surface of almost all clinical isolates of *S. epidermidis* we have examined and have documented opsonic activity of antibody raised against strain RP62A PS/A for several other staphylococcal strains. Thus, these immunological principles should be generally applicable to the prevention of *S. epidermidis* prosthetic valve endocarditis.

The rabbit model of endocarditis used in these experiments differs from rabbit and rodent models used by previous investigators in two respects.[33–35] First, the bacterial challenge occurred 7 days after insertion of the left ventricular catheter rather than after a 24- to 48-hour interval. More importantly, a relatively low, persistent bacterial challenge was delivered by a contaminated catheter in the jugular vein rather than by one-time injection of a very high bacterial inoculum through a peripheral vein. Our method was found to produce endocarditis very reliably while exposing the left ventricular catheter and aortic valve to levels of organisms more likely to be encountered in clinical practice. Indeed, in order to establish coagulase-negative staphylococcal endocarditis by bolus injection of microorganisms, a dose of 10^9 cfu/rabbit was needed, clearly well above any level of exposure a patient might receive. Not surprisingly, this level of challenge dose overwhelmed host defenses when we tried to protect rabbits by immunization with PS/A. We also found that endocarditis was not produced if the catheter in the aortic valve was removed before bacterial challenge (data not shown). Thus, it seems likely that infection of the catheter itself occurs initially, followed by infection of the adjacent valve leaflets. Therefore, this model may mimic some aspects of prosthetic valve endocarditis because of the need for a foreign body in the heart in order to establish infection by coagulase-negative staphylococci.

Native heart valves are relatively resistant to infection with coagulase-negative staphylococci. Although coagulase-negative staphylococcal endocarditis clearly can occur on damaged native valves,[36–38] such infections are uncommon. In contrast, prosthetic heart valves of all types are prone to infection with coagulase-negative staphylococci, especially *S. epidermidis*, which is the most frequent cause of prosthetic valve endocarditis.[1–8] The vast majority of cases of *S. epidermidis* endocarditis that occur in the 12-month period following surgery appear to be nosocomial in origin.[6,7,30] Inoculation of staphylococci probably occurs most frequently in the operating room. The principal source of *S. epidermidis* is the patient's own skin flora, but carriers of *S. epidermidis* on the surgical staff[39,40] and contaminated cardiopulmonary bypass blood[41] have also been implicated by careful typing of bacterial isolates. Some valves may be seeded as a result of intravascular catheter-associated bacteremia, which occurs frequently in the immediate postoperative period.

Regardless of the specific source of the microorganisms that infect prosthetic valves, *S. epidermidis* endocarditis is very difficult to treat. Infection frequently involves the valve ring and adjacent myocardium, leading to dehiscence or valve dysfunction and conduction abnormalities.[7] In addition, the majority of strains are methicillin-resistant, which considerably complicates antibiotic therapy.[7] Not surprisingly, surgical removal of the infected valve is ultimately required in most cases.[7,30] Thus, prevention of infection is of paramount importance. Unfortunately, the usual cephalosporin regimens used for antibiotic prophylaxis are ineffective against methicillin-resistant strains of staphylococci. Resistance to vancomycin, the most commonly used alternative antibiotic, has already been reported.[42] Inoculation of the heart valve might be avoided in many patients by scrupulous attention to surgical aseptic technique, but even the most experienced surgical teams have been unable to prevent this complication entirely.

In conclusion, immunoprophylaxis targeted at the capsular PS/A of *S. epidermidis* may provide a promising new approach to the control of staphylococcal infections of prosthetic heart valves and other implantable medical devices.

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KEY WORDS • coagulase-negative *Staphylococcus* • prostheses • immunity

Appendix D

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Relationship between Surface Accessibility for PpmA, PsaA, and PspA and Antibody-Mediated Immunity to Systemic Infection by *Streptococcus pneumoniae*

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Antibodies to capsular polysaccharide (PS) are protective against systemic infection by *Streptococcus pneumoniae*, but the large number of pneumococcal serogroups and the age-related immunogenicity of pure PS limit the utility of PS-based vaccines. In contrast, cell wall-associated proteins from different capsular serotypes can be cross-reactive and immunogenic in all age groups. Therefore, we evaluated three pneumococcal proteins with respect to relative accessibility to antibody, in the context of intact pneumococci, and their ability to elicit protection against systemic infection by encapsulated *S. pneumoniae*. Sequences encoding pneumococcal surface adhesin A (PsaA), putative protease maturation protein A (PpmA), and the N-terminal region of pneumococcal surface protein A (PspA) from *S. pneumoniae* strain A66.1 were cloned and expressed in *Escherichia coli*. The presence of genes encoding PsaA, PpmA, and PspA in 11 clinical isolates was examined by PCR, and the expression of these proteins by each strain was examined by Western blotting with antisera raised to the respective recombinant proteins. We used flow cytometry to demonstrate that PspA was readily detectable on the surface of the pneumococcal strains analyzed, whereas PsaA and PpmA were not. Consistent with these observations, mice with passively or actively acquired antibodies to PspA or type 3 PS were equivalently protected from homologous systemic challenge with type 3 pneumococci, whereas mice with passively or actively acquired antibodies to PsaA or PpmA were not effectively protected. These experiments support the hypothesis that the extent of protection against systemic pneumococcal infection is influenced by target antigen accessibility to circulating host antibodies.

Streptococcus pneumoniae is a leading cause of morbidity and mortality in developed and developing countries (38). Each year *S. pneumoniae* causes approximately 1.2 million deaths worldwide from pneumonia (43). Antibiotics are effective at controlling many cases of pneumococcal infection, but their use does not prevent mortality within the first 48 h of presentation. The effectiveness of therapeutic care is further constrained by the widespread occurrence of antibiotic-resistant pneumococcal strains (15, 16), and several retrospective studies have reported essentially no change in fatality rates due to pneumococcal bacteremia over the past 40 to 60 years (2, 26). These factors have stimulated renewed interest in the prevention of pneumococcal infections by using vaccines.

Prophylactic vaccines based on capsular polysaccharides (PS) of the pneumococcus are currently the only licensed vaccines available against *S. pneumoniae*. The 23-valent PS vaccine is not effective in children younger than 5 years (12), whereas the recently licensed 7-valent conjugate vaccine only covers a limited number of pneumococcal serotypes (18). The effectiveness of the 7-valent conjugate vaccine at reducing systemic pneumococcal disease due to vaccine serotypes and cross-reactive strains is well established (4, 50). However, this effectiveness of the conjugate vaccine is partially counterbalanced by recent reports documenting increases in pneumococcal disease due to non-vaccine-related serotypes (14, 33). This serotype replacement phenomenon has stimulated interest in developing vaccine strategies aimed at controlling pneumococcal disease in a non-serotype-restricted manner. A number of pneumococcal proteins that function as virulence factors have been identified and characterized as potential vaccine targets for inclusion in a universal pneumococcal vaccine (22). Several of these virulence factors, including PsaA (42), PpmA (36), and PspA (7), have been shown to be cell-wall-associated proteins expressed by all strains of *S. pneumoniae* analyzed to date. The genes for PsaA, PpmA, and PspA and their corresponding proteins have each been characterized in multiple pneumococcal strains. From these studies, the general observation was made that PsaA and PpmA are highly conserved, whereas PspA is relatively more variable at the DNA and protein sequence levels, among pneumococcal strains. We recently reported that immunization of mice with PsaA was only modestly protective against lethal systemic pneumococcal infection and that this relatively limited vaccine efficacy was correlated with inaccessibility of antibodies to PsaA on the surface of an intact encapsulated *S. pneumoniae* type 3 strain (17).

We undertook the present studies to increase our understanding of the relationship between accessibility to antibodies of potential vaccine targets on a diverse panel of pneumococcal strains and ability to elicit protective antibodies. We describe the accessibility of the cell-wall-associated proteins PsaA, PpmA, and PspA in 12 pneumococcal strains. We also assess the ability of active immunization with recombinant forms of PsaA, PpmA, or PspA, or passive immunization with poly-

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TABLE 1. Strains of *S. pneumoniae* used in this study

Strain	Capsule type	Comments	Source[a]
A-69	1	Clinical isolate	M. Jacobs
A-70	2	Clinical isolate	M. Jacobs
2001-169-0205	3	Sputum isolate	M. Jacobs
A66.1	3	Laboratory strain[b]	D. Briles
60161	4	Ear isolate	M. Jacobs
40206	5	Ear isolate	M. Jacobs
E-68	6B	Ear isolate	M. Jacobs
CP-0105	9V	Ear isolate	M. Jacobs
E-70	14	Ear isolate	M. Jacobs
98-105-0105	18C	Ear isolate	M. Jacobs
CP-0075	19F	Ear isolate	M. Jacobs
E-69	23F	Ear isolate	M. Jacobs

[a] Pneumococcal isolates were obtained from the collections of Michael Jacobs (Case Western Reserve University, Cleveland, Ohio) and David Briles (University of Alabama, Birmingham) as indicated.

[b] Strain A66.1 (capsular type 3) is a derivative of clinical isolate A66 (3). The virulence of this strain has been maintained by laboratory passage in mice for more than 30 years (David Briles, unpublished results).

TABLE 2. Sequences of oligonucleotide primers used for PCR amplification and cloning

Gene	Primer[a]	Sequence (5′-3′)[b]
psaA	21(F)	AATCGTCATATGGCCATGGGCgctagcggtaaa aaagatgcagcttc
	308(R)	ATTCCCCTCGAGAAGCTTGGATCCtgccaatcc ttcagcaatctt
ppmA	22(F)	AATCGTCATATGGCCATGGGCtcgaaaggtcag aaggtgcagac
	313(R)	ATTCCCCTCGAGAAGCTTGGATCCttcgttgat gtactactgcttgagc
pspA	26(F)	AATCGTCATATGGCCATGGGCcctactttttgtaag agcagaagagtg/tc
	409(R)	ATTCCCCTCGAGAAGCTTGGATCCacctgtttct tgttttcagcc

[a] Numbers represent the first amino acid encoded by the forward (F) primers or the last amino acid encoded by the reverse (R) primers, respectively. All numbering is based on pre-pro amino acid sequences for each gene.

[b] Restriction sites in each primer relevant to this study (NcoI for "F" primers and XhoI for "R" primers) are in boldface; nucleotides in lowercase are derived from sequences of each gene deposited in GenBank.

clonal antisera raised against these proteins, to protect mice against lethal systemic pneumococcal infection. The implications of our results for pneumococcal vaccine design based on highly conserved surface proteins are discussed.

MATERIALS AND METHODS

Mice. Six- to eight-week-old BALB/c mice were housed under specific-pathogen-free conditions and given sterile food and water ad libitum. The mice were purchased from Taconic Farms, Germantown, N.Y. The Case Western Reserve University Institutional Animal Care and Use Committee approved all animal experiments.

Bacteria. *Escherichia coli* DH5α (Invitrogen) was used as the host for routine plasmid cloning. Recombinant proteins were expressed in *E. coli* BL21(DE3)/pLysS (Novagen, Inc., Madison, Wis.). *E. coli* were cultured in Luria broth supplemented with antibiotics. Virulent *S. pneumoniae* strain A66.1 (3, 6) was used for challenge experiments and as a source of genomic DNA for PCR amplification experiments. Clinical isolates of *S. pneumoniae*, including serotypes responsible for the majority of pneumococcal infections in the United States (25), were selected from a library of approximately 10,000 independent isolates at the University Hospitals of Cleveland, Cleveland, and are listed in Table 1. *S. pneumoniae* were routinely grown on Trypticase soy agar plates supplemented with 5% sheep blood (blood agar) or in Todd-Hewitt broth supplemented with 0.5% yeast extract (Difco, Detroit, Mich.).

Production of recombinant PsaA, PpmA, and PspA. The production of recombinant PsaA, PpmA, and PspA was achieved by PCR amplification of pneumococcal genes, with subsequent cloning and expression of the genes in *E. coli*. Oligonucleotide primers used in PCR amplification experiments were all purchased from Life Technologies, Bethesda, Md., and are listed in Table 2. Pneumococcal genes used for protein expression were amplified from genomic DNA of *S. pneumoniae* strain A66.1 by using the high-fidelity thermostable DNA polymerase, Platinum *Pfx* (Life Technologies). The coding sequence for mature, unmodified, mature PsaA was amplified with the primers PsaA 21(F) and PsaA 308(R); the coding sequence for unmodified, mature PpmA was amplified with the primers PpmA 22(F) and PpmA 313(R), and the coding sequence corresponding to the mature N-terminal region of PspA including the first of the choline-binding repeats (32) was amplified by using PspA 26(F) and PspA 409(R). The coding sequences for PsaA, PpmA, and PspA used for protein expression were cloned into plasmid pET29b+ (Novagen) at the NcoI and XhoI sites, with *E. coli* DH5α as the bacterial host. Each recombinant protein is flanked by a plasmid-encoded N-terminal S tag and a C-terminal polyhistidine tag. For recombinant protein expression, each recombinant pET29 plasmid was transformed into the *E. coli* expression strain BL21(DE3)/pLysS. Recombinant protein expression was initiated by induction with IPTG (isopropyl-β-D-thiogalactopyranoside) and proteins were purified from the soluble fraction of recombinant *E. coli* lysates by using metal affinity chromatography resin and buffers (Novagen), according to the manufacturer's instructions. Protein concentrations were estimated by using the Bradford kit from Bio-Rad (Hercules, Calif.). The recombinant proteins were filter sterilized (Millipore) and stored at 4°C.

Detection of genes encoding pneumococcal cell-wall-associated proteins. PCR amplification was used to demonstrate the presence of genes encoding PsaA, PpmA, and PspA in clinical isolates of *S. pneumoniae*. For this purpose, genomic DNAs were prepared from 11 pneumococcal strains by using a genomic DNA isolation kit (Qiagen) and were used as templates for PCR amplification with *Taq* polymerase (Fisher) with the primers listed in Table 2. Amplification products were electrophoresed through 1% agarose gels and visualized by staining with ethidium bromide (0.5 μg/ml).

Production of hyperimmune mouse sera against pneumococcal antigens. Hyperimmune mouse sera specific for PsaA (anti-PsaA), PpmA (anti-PpmA), or PspA (anti-PspA) were generated by intraperitoneal (i.p.) immunization of mice with each recombinant protein emulsified in incomplete Freund's adjuvant (IFA) (1:1 ratio [vol/vol]). Sera specific for type 3 PS (anti-PS) were generated by inoculating mice i.p. twice at 10-day intervals with type 3 PS (obtained from the American Type Culture Collection) in phosphate-buffered saline (PBS). Pooled sera prepared from blood collected 2 weeks after the final immunization were stored at −20°C until used for assays.

Detection of antibodies to pneumococcal antigens. The levels of antibodies specific for PsaA, PpmA, or PspA in sera from immunized mice were monitored by enzyme-linked immunosorbent assay (ELISA), as previously described (17). Immulon 1 plates (Dynatech, Chantilly, Va.) were coated with recombinant PsaA, PpmA, or PspA (10 μg/ml, 100 μl per well in PBS) overnight at 4°C. Individual sera from immunized mice were tested in duplicate. The binding of antibodies to their cognate antigens was detected by using alkaline phosphatase-conjugated goat anti-mouse immunoglobulins (subclass specific, Southern Biotechnologies, Birmingham, Ala.), followed by incubation in *p*-nitrophenyl phosphate (Sigma). Antibody titers were determined as the highest dilution of serum giving a detectable absorbance reading above background. Background in all of the ELISAs was defined as the mean absorbance values for sera obtained from mice unimmunized with mouse serum albumin (MSA) diluted 1 to 100 in PBS. These background absorbance values were close to zero throughout all of the experiments performed and were arbitrarily assigned a titer of 1:100 for each respective antigen tested by ELISA. Antibody titers specific for type 3 PS were determined in a similar fashion by using Polysorp plates (Nunc, Roskilde, Denmark) coated with type 3 PS (10 μg/ml, 100 μl per well) overnight at 4°C, as previously described (27). Serial dilutions of sera were tested in duplicate. Our observation that MSA-immunized mice exhibited low background absorbances to each of the pneumococcal antigens tested by ELISA provided additional evidence that the cohorts of mice evaluated in these experiments had not previously been exposed to *S. pneumoniae*.

Detection of pneumococcal protein expression by polyacrylamide gel electrophoresis and Western blot analysis. Recombinant PsaA, PpmA, PspA, and whole-cell lysates of *S. pneumoniae* strains and *Salmonella enterica* serovar Typhimurium or *E. coli* BL21(DE3)pLysS as a negative control were subjected

to sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE) and electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad) for Western blot analysis. Individual blots were reacted with hyperimmune serum specific for either PsaA, PpmA, or PspA. The membranes were subsequently incubated in alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG; γ-chain specific, Southern Biotechnologies) and developed by incubation in BCIP (5-bromo-4-chloro-3-indolylphosphate)-nitroblue tetrazolium (NBT) chromogenic phosphatase substrate (Sigma).

Detection of surface expression of pneumococcal proteins. Indirect immunofluorescence was carried out to determine the ability of antibodies raised against recombinant pneumococcal antigens to bind to the surface of intact *S. pneumoniae*, as previously described (17). Cryopreserved bacteria corresponding to 12 pneumococcal isolates were streaked individually onto blood agar plates incubated for ~12 h at 37°C. Bacteria were harvested from the plates, washed in sterile PBS, and resuspended in staining buffer (PBS with 0.05% sodium azide and 1% bovine serum albumin). Approximately 2×10^7 bacteria were incubated with 10% serum from mice inoculated with MSA as negative controls or specific antigens (PS, PsaA, PpmA, or PspA). After incubation at 4°C, bacteria were washed in staining buffer and incubated with a 1:50 dilution in staining buffer of a $F(ab')_2$ fragment of goat anti-mouse IgG (H+L) conjugated to Alexa 488 fluorescent dye (Molecular Probes Inc., Eugene, Oreg.). Bacteria were then washed in PBS and subjected to flow cytometry using a Becton Dickinson Bench-top flow cytometer. The data were collected and analyzed by using CellQuest software (Becton Dickinson).

PspA typing of clinical isolates. Currently available data indicate that PspAs among pneumococcal strains can be divided into three families. DNA sequence analysis has been used to assign PspAs from different isolates to family 1 (44%) and family 2 (55%) with a minority (~1%) of PspAs being assigned to family 3 (7, 19). PspAs are highly cross-reactive (10), but by analysis with well-chosen or with absorbed sera, it is possible to distinguish PspAs of family 1 and family 2 by their relative reactivities with a pair of antisera made against reference family 1 or family 2 proteins (48). In these studies, antisera relatively specific for family 1 and 2 PspA were used, and the reactivities of pneumococcal lysates with the anti-family 1 and anti-family 2 sera were determined by dot blots, as previously described (48). For dot blot analysis, serial dilutions of pneumococcal lysates were spotted onto each of two nitrocellulose membranes. After blocking of excess binding sites with blocking buffer (PBS containing 1% bovine serum albumin and 0.05% Tween 20), the membranes were incubated in 1:5,000 dilutions of pooled polyclonal rabbit antisera raised against PspA from strains Rx1 and 1282H0 (corresponding to family 1), or pooled polyclonal rabbit antisera raised against PspA from strains V-024 and V-032 (corresponding to family 2). After washes, the membranes were incubated sequentially with biotinylated goat-anti-rabbit IgG and streptavidin conjugated to alkaline phosphatase. Color was developed by using BCIP-NBT chromogenic phosphatase substrate.

PCR was used to confirm the PspA families by using genomic DNA of strains that reacted equally well with PspA family 1 and family 2 polyclonal rabbit antisera in the dot blot assay described above. Oligonucleotide primers LSM12 and SKH63 were used to detect family 1 PspA coding sequences, and primers LSM12 and SKH52 were used to detect family 2 PspA coding sequences, respectively, as previously described (26, 48).

Pneumococcal challenge of actively immunized mice. BALB/c mice to be used in challenge experiments were primed with 250 pmol of either PsaA or PpmA (~9.3 μg) or 100 pmol of PspA (~5 μg), each in complete Freund's adjuvant (1:1 ratio [vol/vol]) on day zero, and boosted with the same concentration of each respective antigen in IFA (1:1 ratio [vol/vol]) on day 14. The amounts of PsaA and PspA used for immunizations were based on doses used to elicit high titers of specific antibody in previous studies (17, 28), and the amount of PpmA used for immunizations was established in preliminary experiments (data not shown). We used higher doses of PsaA and PpmA, relative to PspA, in order to compensate for the higher immunogenicity of PspA, which became apparent in preliminary studies. BALB/c mice immunized with 0.5 μg of type 3 PS in sterile PBS on days 0 and 14 served as positive controls, and mice injected with 1% MSA in sterile PBS served as negative controls. The amount of PS used was based on previous studies by us demonstrating that this dose resulted in a protective type 3 PS-specific antibody response in BALB/c mice (17, 29). All vaccines were administered i.p. All mice were bled on days 10 and 21 and challenged on day 25. Individual sera from each immunized mouse were tested for the presence of specific antibodies prior to challenge with live pneumococci. Virulent type 3 *S. pneumoniae* (strain A66.1) grown to log phase was prepared for challenge via the i.p. route in actively immunized mice, as previously described (17). For challenge infections, mice were injected i.p. with approximately 500 CFU of virulent *S. pneumoniae* strain A66.1 (type 3) suspended in PBS. The actual number of CFU administered was determined retrospectively by plating serial dilutions of the inocula on blood agar. The survival of mice was monitored for 15 days, at which time the experiments were terminated.

Pneumococcal challenge of passively immunized mice. Two types of passive immunization and challenge experiments were performed. In the first series of experiments, the groups of four to five mice to be challenged were passively immunized with 100 μl of hyperimmune serum specific for PsaA, PpmA, PspA, or type 3 PS (prepared as described above) by i.p. injection. At 24 h after passive immunization, each mouse was challenged intraperitoneally with approximately 1,000 CFU of virulent A66.1 pneumococci suspended in PBS, and survival was monitored for 15 days. In a second series of experiments, groups of mice were inoculated with 1,000 CFU of A66.1 suspended in 100 μl of PBS containing 10% hyperimmune serum specific for PsaA, PpmA, PspA, or type 3 PS in PBS. Survival of mice was monitored for 15 days.

Statistical analysis. The Fisher exact test was used to compare overall survival rates for mice immunized with MSA to those of mice immunized with PsaA, PpmA, PspA, or type 3 PS. The same statistical analyses were performed to evaluate differences in overall survival rates for mice passively immunized with pooled sera from MSA-immunized mice versus mice passively immunized with pooled immune sera specific for PsaA, PpmA, PspA, or type 3 PS. Values were considered statistically significant at a P value of <0.05 (two-tailed).

RESULTS

Presence of selected pneumococcal genes in *S. pneumoniae* isolates. PCR amplification was used to demonstrate the presence of genes encoding the pneumococcal proteins PsaA, PpmA, and PspA in 12 isolates of *S. pneumoniae* (Fig. 1). The three genes demonstrated the range of variability known to exist for nucleotide sequences encoding pneumococcal surface proteins. Bands corresponding to PsaA, PpmA, and PspA were detected in all strains of *S. pneumoniae* analyzed. PCR amplification with primers specific for PsaA and PpmA exhibited single bands of identical size (ca. 900 bp for each gene) in all strains, while PCR amplification with PspA specific primers exhibited bands of different sizes from the different *S. pneumoniae* strains, although 50% of the strains showed a predominant band approximately 1.2 kb in size. These results support the notion that PsaA and PpmA are highly conserved at the DNA level, whereas the PspA locus exhibits the previously reported size variability from strain to strain (19, 48).

Expression and characterization of recombinant pneumococcal proteins. All three recombinant proteins were recovered in the soluble fraction of the *E. coli* expression strains and were purified to near homogeneity by metal affinity chromatography. Recombinant PsaA, PpmA, and PspA were characterized by SDS-PAGE (Fig. 2). PsaA and PpmA migrated in SDS-PAGE gels according to their predicted molecular masses (ca. 37 kDa for each protein). rPspA appeared to be larger than its predicted molecular mass (75 kDa versus an expected mass of 47 kDa). The reason for the lack of concordance between the apparent and actual sizes of PspA is not known but has been previously described for other PspA genes expressed by *S. pneumoniae* (53), as well as a recombinant PspA gene fragment expressed by *S. enterica* serovar Typhimurium (32). Each protein was used to prepare polyclonal mouse antisera by repeated inoculation of mice with each respective antigen emulsified in IFA for use in subsequent immunoassays.

Characterization of protein expression in pneumococcal isolates. Western blots were used to demonstrate the expression of genes encoding PsaA, PpmA, and PspA in lysates of the *S. pneumoniae* strains listed in Table 1. Antisera specific for PsaA or PpmA reacted with a single band of ca. 35 kDa in lysates of all of the strains of *S. pneumoniae* tested (Fig. 3A and B). The

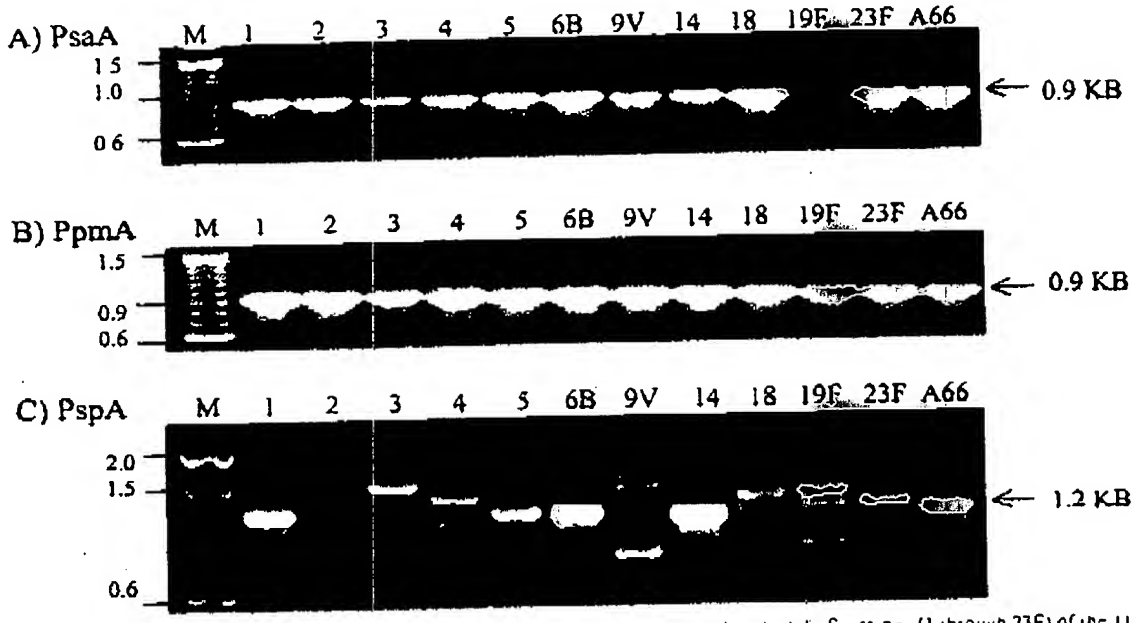

FIG. 1. PCR analysis of *S. pneumoniae* strains. Molecular weight markers are indicated at the left. Serotypes (1 through 23F) of the 11 isolates (Table 1) from which genomic DNA was amplified are indicated; A66 refers to capsular type 3 strain A66.1. All PCRs were performed by using *Taq* polymerase under identical conditions (5 min at 95°C, followed by 30 cycles of 95°C for 30 s, 52°C for 45 s, and 72°C for 2 min, and finally 10 min at 72°C. M, 100-bp DNA ladder (BRL). Arrows at the right indicate the gene fragments of PsaA, PpmA, or PspA amplified from strain A66.1 by using high-fidelity *Pfx* polymerase (BRL), which were cloned and expressed in *E. coli*. KB, kilobase(s).

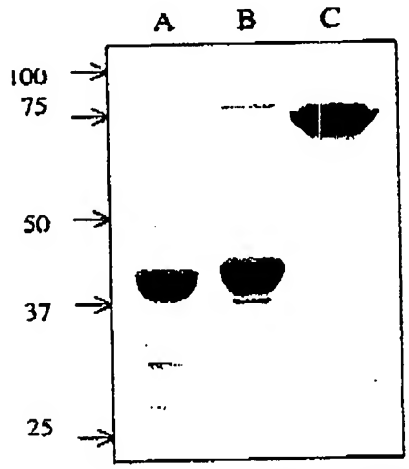

FIG. 2. Recombinant PsaA (A), PpmA (B), and PspA (C) proteins from *S. pneumoniae* strain A66.1 were cloned, expressed, and purified from *E. coli* lysates by metal affinity chromatography. The proteins (4 μg per lane) were subjected to SDS-PAGE and detected by direct staining with Coomassie brilliant blue. Apparent molecular size markers (in kilodaltons) are indicated. These purified recombinant proteins were used to prepare mouse polyclonal antisera specific for PsaA, PpmA, and PspA, respectively, for use in subsequent immunological analyses.

antisera did not react with a lysate of *S. enterica* serovar Typhimurium which was included as a negative control or with a lysate of the untransformed *E. coli* expression strain from which the recombinant proteins were purified (data not shown). It was clear that the two antisera did not cross-react with noncognate molecules since Western blots of the recombinant proteins (PsaA and PpmA) showed no cross-reactivity using the same antisera (data not shown).

The PspA-specific antiserum reacted with several bands in each *S. pneumoniae* lysate (Fig. 3C). The PspA-specific antiserum did not react with a lysate of *S. enterica* serovar Typhimurium or with a lysate of the untransformed *E. coli* expression strain from which the recombinant proteins were purified (data not shown).

Our observation that the PspAs of different strains are of different sizes is consistent with previous results (10, 49). These differences are in large part due to large differences in open reading frames of different PspAs (19). In the present study and in previous studies it has been observed that individual PspAs can yield multiple bands. These additional bands are due in part to the fact that some of the PspA molecules from some strains migrate in the SDS gel as dimers, while the rest migrate as monomers (44). The heterogeneity in the size of PspA from a single strain is also thought to result from limited proteolytic cleavage that inevitably occurs during sample preparation (44). There are also data that, under some circumstances, there can be some cross-reactivity between PspA and PspC, which may result in additional apparent heterogeneity (9). Another anomaly with PspA migration on SDS gels is that

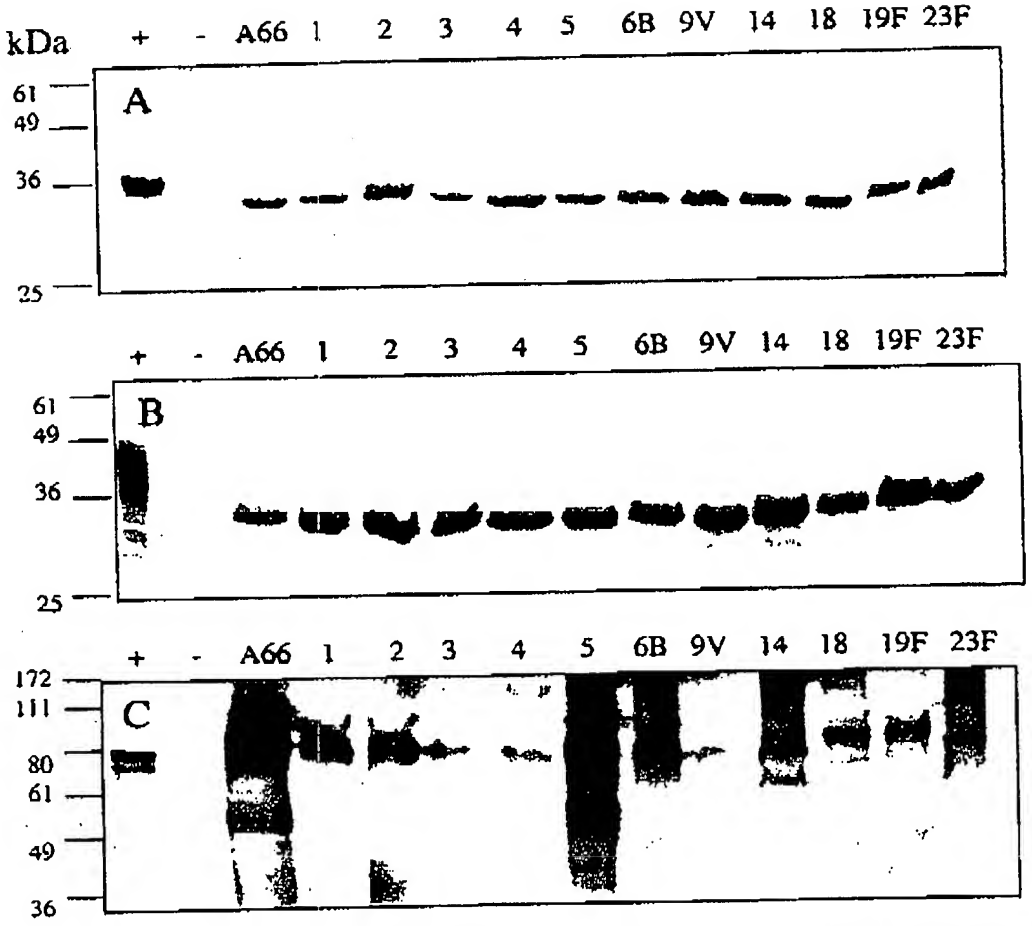

FIG. 3. Western blot analysis of *S. pneumoniae* strains. SDS–10% polyacrylamide gels were loaded with rPsaA (A), rPpmA (B), or rPspA (C) (all derived from strain A66.1 coding sequences) as positive controls (+), *S. enterica* serovar Typhimurium lysate as a negative control (A to C, −), and *S. pneumoniae* lysates from strain A66 and the strains described in Table 1 and indicated by their serotypes. Electrophoresed proteins were transferred to polyvinylidene difluoride membranes and incubated with polyclonal anti-PsaA (A), anti-PpmA (B), or anti-PspA (C). Blots were developed with alkaline phosphatase-conjugated goat anti-mouse IgG (γ-chain specific) and visualized by incubation in BCIP-NBT chromogenic substrate. Apparent molecular size markers in kilodaltons are indicated.

the PspA monomer apparently retains enough rigidity that it commonly runs somewhat larger than would be predicted by its actual molecular mass (53).

Surface expression of antigens in intact *S. pneumoniae*. We were interested in investigating the ability of sera raised against select pneumococcal surface antigens to bind to the surface of intact *S. pneumoniae*. Initial comparison of the surface binding of anti-PsaA, anti-PpmA, anti-PspA, or anti-PS to *S. pneumoniae* strain A66.1 by flow cytometry confirmed our previous finding (17) that PsaA was not detected on the surface of *S. pneumoniae* strain A66.1, while the binding of anti-PS was readily detected on the surface of this strain (Fig. 4). In addition, the binding of anti-PspA to the surface of strain A66.1 was readily detected, whereas anti-PpmA did not exhibit any apparent binding to the surface of strain A66.1 (Fig. 4).

We subsequently used the same surface immunofluorescence assay to demonstrate that neither PsaA nor PpmA are accessible to antibodies on the surface of 11 clinical isolates of *S. pneumoniae* of the indicated serotypes (Fig. 4). In contrast, PspA was readily detectable on the surface of 11 of the 11 clinical isolates of *S. pneumoniae* tested (Fig. 4). The low level of binding of anti-PspA to the surfaces of the types 2 and 3 *S. pneumoniae* strains in the present study could be the result of the known heterogeneity in primary sequences of PspA that can result in a low level of cross-reactivity of some PspAs with an antiserum raised to a single PspA (45, 48) (in this case a family 1 PspA from strain A66.1). This interpretation appears to be supported by our demonstration that the PspA genes in these two strains belong to family 2 (Table 3), which is generally only weakly cross-reactive with antibodies raised against family 1 PspA (48). Taken together, these surface immunofluorescence studies confirm that PspA is highly accessible to antibodies at the surface of the intact pneumococcus (1), in

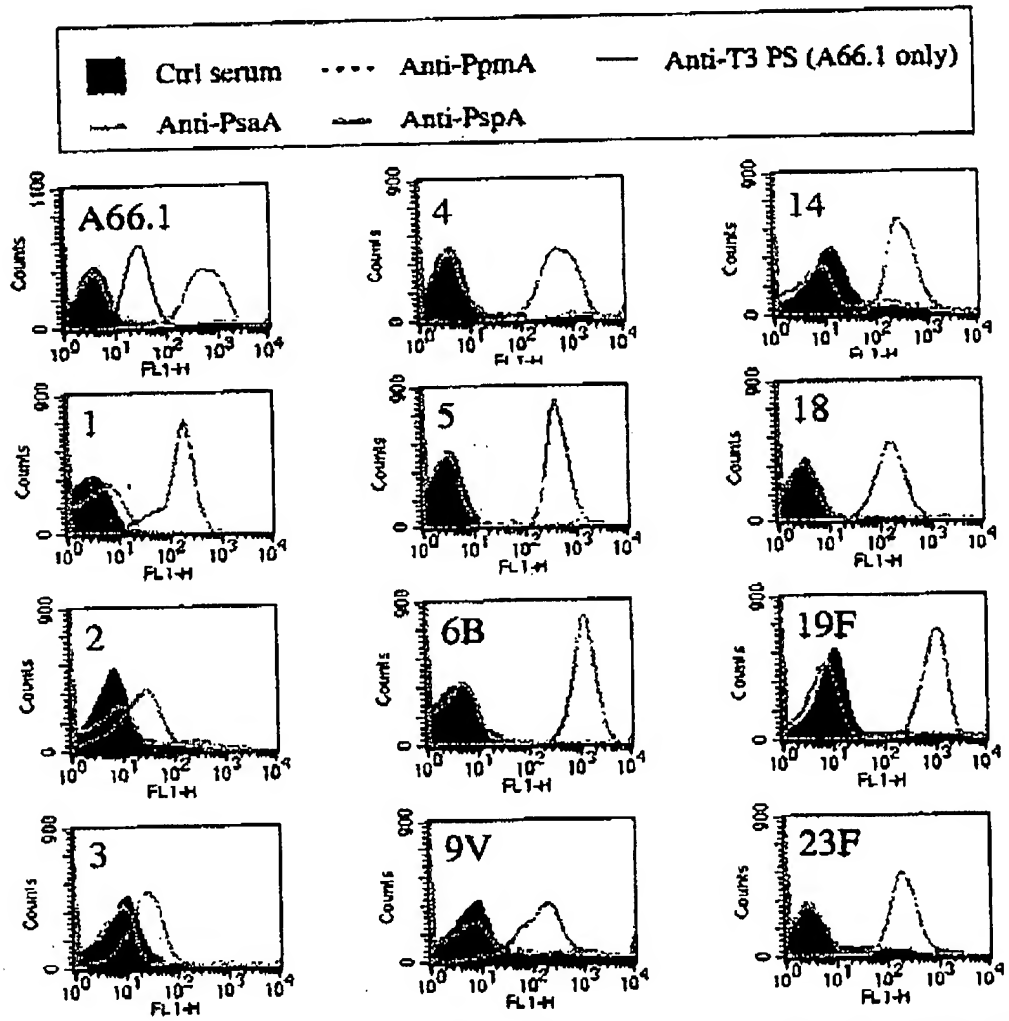

FIG. 4. Flow cytometric analysis of *S. pneumoniae* isolates. Bacteria were incubated with either control serum, anti-type 3 PS (strain A66.1 only), anti-PsaA, anti-PpmA, or anti-PspA, followed by incubation with a $F(ab')_2$ fragment of goat anti-mouse IgG (H+L) conjugated to Alexa 488 fluorophore. Bacteria were analyzed by flow cytometry using side scatter as the threshold for detection. Specific binding by antisera is indicated as log fluorescence intensity on the *x* axis (FL1-H). Each histogram represents 100,000 events (bacterium-sized particles).

a fashion analogous to capsular PS, whereas PsaA and PpmA are not readily accessible to antibodies under similar experimental conditions.

Protection of BALB/c mice from lethal systemic infection with *S. pneumoniae*. In order to determine whether the accessibility of antigen to antibodies, as assessed by flow cytometry, predicts ability to elicit protective humoral immunity, a series of challenge experiments were performed. In the first series of experiments, mice actively immunized with pneumococcal surface antigens were challenged i.p. with ca. 500 CFU of *S. pneumoniae* strain A66.1 (type 3). Mice immunized with MSA served as negative controls, and mice immunized with type 3 PS served as positive controls. Mice immunized with either PspA or the homologous type 3 PS were significantly protected, whereas mice immunized with either PsaA or PpmA were not effectively protected from systemic challenge with virulent *S. pneumoniae* (Table 4). Sera obtained from immunized mice 3 days before challenge with live pneumococci were individually tested by ELISA for the presence of specific antibody to the respective antigens used for immunization. These data confirmed that each mouse had high titers of antibodies for each of the pneumococcal antigens administered (data not shown). To demonstrate that the observed protection was antibody mediated, groups of naive mice were passively immunized with anti-MSA, anti-PsaA, anti-PpmA, anti-PspA, or anti-PS, either 24 h prior to challenge or at the time of challenge with virulent *S. pneumoniae* strain A66.1 grown to log phase. The results were similar whether the mice received

TABLE 3. PspA typing of *S. pneumoniae* isolates used in this study

Strain[a]	Capsule type	PspA family	
		Dot blot[b]	PCR[c]
A-69	1	1	ND
A-70	2	2	ND
2001-169-0203	3	2	ND
A66.1	3	1	ND
80101	4	1, 2	2
40206	5	1	ND
E-68	6B	1	ND
CP-0105	9V	2	ND
E-70	14	1	ND
98-105-0105	18C	1, 2	2
CP-0075	19F	1, 2	2
E-69	23F	1	ND

[a] From Table 1

[b] PspA families were assigned to the various pneumococcal isolates on the basis of reactivity with polyclonal rabbit antisera raised against prototypical family 1 and family 2 PspAs.

[c] PCR was used to confirm the PspA family for PspAs from pneumococcal strains that reacted equally well with antisera raised against prototypical family 1 and family 2 PspAs. ND, not determined

TABLE 5. Protection of BALB/c mice after passive immunization with immune serum to pneumococcal antigens[a]

Serum specificity	Titer[b]	No. alive/ total no.[c]	% Survival	P (versus MSA-immunized mice)[c]
Ctrl[d]	≤100	1/10	10	NA
PsaA	218,700	0/12	0	0.45
PpmA	72,900	0/12	0	0.45
PspA	140,929	9/12	75	0.0034*
T3 PS	6,100	10/12	83	0.0009*

[a] Naive BALB/c mice were challenged with 1,000 CFU of live *S. pneumoniae* strain A66.1 (type 3) i.p. after passive immunization with pooled immune serum specific for the indicated pneumococcal antigens

[b] Control (Ctrl) mice were given *S. pneumoniae* after passive immunization with pooled serum from mice inoculated with MSA

[b] Antibody titers for each specific antigen in pooled immune sera were measured by ELISA. Absorbance values for MSA-immunized mice were arbitrarily assigned a relative titer for reactivity to each respective antigen of ≤100

[c] Survival of mice was monitored for 15 days after challenge with *S. pneumoniae*. *, Statistically significant overall survival ($P < 0.05$) compared to the overall survival for control mice, as calculated by using the Fisher exact test. NA, not applicable.

serum at the time of challenge or 24 h prior to challenge and have therefore been combined in Table 5. Only mice that received anti-PspA or anti-PS were significantly protected against homologous challenge with virulent *S. pneumoniae* strain A66.1, whereas mice that received anti-PsaA, anti-PpmA, or pooled sera from MSA-immunized mice were not protected against challenge with *S. pneumoniae* strain A66.1. These passive immunization experiments suggest a direct relationship between antibody accessibility to antigens on the pneumococcal surface and protection against systemic pneumococcal infection.

DISCUSSION

Antibodies to capsular PS represent the de facto "gold standard" for vaccines against *S. pneumoniae* infection. Antibodies against capsular PS are highly protective against invasive pneumococcal disease and, when present at the mucosal surface, appear also to be effective at reducing the carriage of homologous or cross-reactive pneumococcal strains (13). The primary host protective mechanism against systemic pneumococcal infection is generally believed to be opsonophagocytosis, which is facilitated by antibodies to surface antigens (24). Based on these observations, we suggest that among suitable candidates for vaccines against pneumococcal invasive disease should be antibody-accessible antigens capable of supporting opsonization, although it is conceivable that protein antigens could elicit antibodies that protect against the pneumococcus on some other basis (e.g., antagonism of protein function or modulation of the inflammatory response). In this regard, it is worth noting that a strategy for the identification of potentially protective antigens based on antibody accessibility at the pneumococcal surface (such as the strategy used in this report) would not pick up protective pneumococcal antigens such as pneumolysin (which is released from the pneumococcus and is not attached to the pneumococcal surface), where the protection appears to be mediated by neutralization of pneumolysin function by antibodies (34, 35).

TABLE 4. Protection of BALB/c mice after active immunization with pneumococcal antigens[a]

Antigen	Amt[b]	No. alive/ total no.[c]	% Survival	P (versus MSA-immunized mice)[c]
Ctrl	NA	1/8	13	NA
PsaA	250 pmol	1/5	20	0.51
PpmA	250 pmol	3/13	23	0.38
PspA	100 pmol	4/5	80	0.031*
T3-PS	0.5 μg	9/9	100	0.0004*

[a] At 2 weeks after the second immunization with the indicated antigens, BALB/c mice were challenged with 500 CFU of live *S. pneumoniae* strain A66.1 (type 3) injected i.p.

[b] Mice were immunized with the indicated antigens on days 0 and 11. T3-PS is type 3 capsular PS. Control (Ctrl) mice were immunized twice with MSA.

[c] Survival of mice was monitored for 15 days after challenge with *S. pneumoniae*. *, Statistically significant overall survival ($P < 0.05$) compared to the overall survival for control mice, as calculated by using the Fisher exact test

Throughout these experiments, we have been guided by the hypothesis that antigens being considered as non-PS pneumococcal vaccine should, after immunization, be able to elicit levels of protection against pneumococcal infection comparable to those generally observed for PS-based vaccines. As such, we used protection provided by immunization with capsular PS as the standard against which to evaluate the protective efficacy of immunization with alternative (non-PS-based) candidate pneumococcal antigens.

It is reasonable to hypothesize that the polymorphism exhibited by certain pneumococcal surface antigens is attributable to immunological selection (19, 31). This hypothesis predicts that surface antigens that exhibit variability from strain to strain are readily accessible to antibodies on the surface of intact pneumococci (such as PspA and PspC, which have been shown to interfere with complement deposition) (21, 37, 47), while highly conserved antigens are generally not readily accessible to antibodies on the surface of the intact pneumococcus. The results of the present study appear to support this hypothesis, since PspA and capsular PS (two examples of antigens that vary from strain to strain) are readily accessible to antibodies in circulation, whereas two more highly conserved antigens (PsaA and PpmA) are not. If

this notion is fundamentally correct, then the ideal third-generation pneumococcal vaccine capable of stimulating protective immunity to the pneumococcus should consist of mixtures of antibody-accessible protein variants from a single locus (such as PspA) or from different loci.

The flow cytometric assay used to assess the surface accessibility of PspA reaffirmed previous observations that although heterogeneity exists among PspAs expressed by different pneumococcal isolates, antibodies raised to a single PspA can cross-react with different PspAs (7, 8). We were able to demonstrate differences in the amounts of PspA-specific antibody that bound to different isolates. These results provide additional support for the hypothesis that the ideal PspA-based subunit vaccine should contain at least one member of each of the major PspA families in order to ensure the elicitation of protective immunity against 90% or more of pneumococci (30, 39, 40, 48).

We noted that relatively low titers of antibody to capsular PS were capable of eliciting a magnitude of protection equivalent to or slightly better than the protection elicited by much higher titers of antibody to PspA in these experiments. Although we did not perform a detailed evaluation of the minimum quantities of PS- or PspA-specific antibodies required to elicit a protective response in these experiments, the flow cytometric assay demonstrated that a larger amount of PspA-specific antibody (which had a high PspA-specific antibody titer by ELISA) bound to the challenge strain (A66.1) than did type 3 PS-specific antibodies, which had a correspondingly low type 3 PS-specific antibody titer, as measured by ELISA.

These data would appear to suggest that the development of PspA as a pneumococcal vaccine should also include strategies aimed at eliciting high titers of PspA specific antibodies. One such strategy would be the genetic fusion of PspA to cytokines, given that immunization of mice with fusion proteins consisting of PspA conjugated to interleukin-2 or granulocyte-macrophage colony-stimulating factor have been shown to dramatically enhance the immunogenicity of PspA (52). In this context, it is worth emphasizing that the advantages offered by protein vaccine antigens, such as PspA, compared to capsular PS reside not in the specific activity of the corresponding antibodies (which are probably lower) but in the prospect of broader serotype coverage and broader age-related immunogenicity.

It is important to note that, although we have demonstrated that PsaA and PpmA are poor vaccine targets for protection against systemic pneumococcal infection (at least under the present experimental conditions) on the basis of their inaccessibility to antibodies, other studies have demonstrated that mucosal immunization of mice with PsaA is highly protective against pneumococcal carriage (5, 7, 11, 23). The exact mechanisms of protection against pneumococcal carriage afforded by immunization with PsaA have not yet been elucidated. A more recent report appears to confirm the importance of immunity to PsaA as being protective against pneumococcal carriage by demonstrating that antibodies against PsaA inhibit the ability of transparent strains of *S. pneumoniae* to adhere to human nasopharyngeal epithelial cells (41).

Two groups have reported the sequencing of the entire pneumococcal genome (20, 46), and another subsequent study reported the discovery of previously unknown surface antigens based on the presence of consensus surface antigen motifs by using a genomic screening approach (51). The suitability of these new antigens as vaccine targets will depend on (among other factors) their variability across pneumococcal strains, as well as their relative accessibility to antibodies in circulation. In the present study we applied a relatively inexpensive method that can be used to screen vaccine candidate antigens, based on their accessibility to antibodies on the surface of intact *S. pneumoniae* (17). The results of these studies should provide insights regarding selection of candidate vaccine targets suitable for inclusion in a universal pneumococcal vaccine, particularly a vaccine designed to protect against systemic pneumococcal infection.

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